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(54) Title: METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS GENE EXPRESSION MODULATORS

(57) Abstract

The present invention provides a method of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene which is expressed by the cell. Molecules useful in the practice of the invention are characterized as follows: (a) do not naturally occur in the cell, (b) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell. Additionally, this invention provides a method for determining whether a molecule known to be a modulator of protein biosynthesis is capable of transcriptionally modulating expression of a gene-of-interest.

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METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION
AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS
GENE EXPRESSION MODULATORS


Background of the Invention

Throughout this application, various publications are
referenced by Arabic numerals within parentheses. Full
citations for these publications may be found at the end
of the specification immediately preceding the claims.
The disclosures of these publications in their entireties
are hereby incorporated by reference into this
application in order to more fully describe the state of
the art as known to those skilled therein as of the date
of the invention described and claimed herein.

The expression of a specific gene can be regulated at any
step in the process of producing an active protein.
Modulation of total protein activity may occur via
transcriptional, transcript-processing, translational or
post-translational mechanisms. Transcription may be
modulated by altering the rate of transcriptional
initiation or the progression of RNA polymerase (28).
Transcript-processing may be influenced by circumstances
such as the pattern of RNA splicing, the rate of mRNA
transport to the cytoplasm or mRNA stability. This
invention concerns the use of molecules which act by

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modulating the _in vivo_ concentration of their target proteins via regulating gene transcription. The functional properties of these chemicals are distinct from previously described molecules which also affect gene transcription.

Researchers have documented the regulation of transcription in bacteria by low molecular weight chemicals (42, 36). Extracellular xenobiotics, amino acids and sugars have been reported to interact directly with an intracellular proteinaceous transcriptional activator or repressor to affect the transcription of specific genes.

Transcriptional regulation is sufficiently different between procaryotic and eucaryotic organisms so that a direct comparison cannot readily be made. Procaryotic cells lack a distinct membrane bound nuclear compartment. The structure and organization of procaryotic DNA elements responsible for initiation of transcription differ markedly from those of eucaryotic cells.

The eucaryotic transcriptional unit is much more complex than its procaryotic counterpart and consists of additional elements which are not found in bacteria. Eucaryotic transcriptional units include enhancers and other cis-acting DNA sequences (30, 19). Procaryotic transcription factors most commonly exhibit a "helix-turn-helix" motif in the DNA binding domain of the protein (29, 37). Eucaryotic transcriptional factors frequently contain a "zinc finger" (37, 12) or a "leucine zipper" (24) in addition to sometimes possessing the "helix-turn-helix" motif (26). Furthermore, several critical mechanisms at the post-transcriptional level such as RNA splicing and polyadenylation are not found in

procaryotic systems (21, 35).

In higher eucaryotes, modulation of gene transcription in response to extracellular factors can be regulated in both a temporal and tissue specific manner (22). For example, extracellular factors can exert their effects by directly or indirectly activating or inhibiting transcription factors (22, 28).

Modulators of transcription factors involved in direct regulation of gene expression have been described, and include those extracellular chemicals entering the cell passively and binding with high affinity to their receptor-transcription factors. This class of direct transcriptional modulators include steroid hormones and their analogs, thyroid hormones, retinoic acid, vitamin D$_3$ and its derivatives, and dioxins, a chemical family of polycyclic aromatic hydrocarbons (12, 38, 9).

Dioxins are molecules generally known to modulate transcription, however, dioxins bind to naturally-occurring receptors which respond normally to xenobiotic agents via transcriptionally activating the expression of cytochrome P450, part of an enzyme involved in detoxification. Similarly, plants also have naturally occurring receptors to xenobiotics to induce defense pathways. For example, the fungal pathogen Phytophthora megasperma induces an anti-fungal compound in soybeans. Such molecules which bind to the ligand binding domains of such naturally occurring receptors are not included on the scope of this invention.

The clinical use of steroid hormones, thyroid hormones, vitamin D$_3$ and their analogs demonstrates that agents

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which modulate gene transcription can be used for beneficial effects, although these agents can exhibit significant adverse side effects. Obviously, analogs of these agents could have similar clinical utility as their naturally occurring counterparts by binding to the same ligand binding domain of such receptors.

Indirect transcriptional regulation involves one or more signal transduction mechanisms. The regulation typically involves interaction with a receptor, the receptor being part of a multistep intracellular signaling pathway, the pathway ultimately modulating the activity of nuclear transcription factors. This class of indirect transcriptional modulators include polypeptide growth factors such as platelet-derived growth factor, epidermal growth factor, cyclic nucleotide analogs, and mitogenic tumor promoters (18, 1, 2).

It is well documented that a large number of chemicals, both organic and inorganic, e.g. metal ions, can non-specifically modulate transcription.

Researchers have used nucleotide analogs in methods to modulate transcription. The mechanism involves incorporating nucleotide analogs into nascent mRNA or non-specifically blocking mRNA synthesis. Similarly, researchers have used alkylating agents, e.g. cyclophosphamide, or intercalating agents, e.g. doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl CoA reductase, e.g. lovastatin, are known to modulate transcription by indirectly increasing expression of hepatic low density lipoprotein receptors as a consequence of lowered cholesterol levels.

Signal effector type molecules such as cyclic AMP,
diacylglycerol, and their analogs are known to
non-specifically regulate transcription by acting as part
of a multistep protein kinase cascade reaction. These
signal effector type molecules bind to domains on
proteins which are thus subject to normal physiological
regulation by low molecular weight ligands (10, 39).

The specific use of sterol regulatory elements from the
LDL receptor gene to control expression of a reporter
gene has recently been documented in PCT/US88/10095. One
aspect of PCT/US88/10095 deals with the use of specific
sterol regulatory elements coupled to a reporter as a
means to screen for drugs capable of stimulating cells to
synthesize the LDL receptor. PCT/US88/10095 describes
neither the concept of simultaneously screening large
numbers of chemicals against multiple target genes nor
the existence of transcriptional modulators which (a) do
not naturally occur in the cell, (b) specifically
transcriptionally modulate expression of the gene-of-
interest, and (c) bind to DNA or RNA or bind to a
protein through a domain of such protein which is not a
ligand binding domain of a receptor which naturally
occurs in the cell, the binding of a ligand to which
ligand binding domain is normally associated with the
defined physiological effect. The main focus of
PCT/US88/10095 is the use of the sterol regulatory
elements from the LDL receptor as a means to inhibit
expression of toxic recombinant biologicals.

The use of molecules to specifically modulate
transcription of a gene-of-interest as described herein
has not previously been reported and its use will bring
surprise since available literature does not propose the
use of a molecule, as described, in a method to

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specifically modulate transcription. Instead, the available literature has reported methods which define domains of transcriptional regulating elements of a gene-of-interest.

Further, the practice of using a reporter gene to analyze nucleotide sequences which regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define domains of transcriptional regulatory elements of a gene-of-interest. Reporter genes which express proteins, e.g. luciferase, are widely utilized in such studies. Luciferases expressed by the North American firefly, Photinus pyralis and the bacterium, Vibrio fischeri were first described as transcriptional reporters in 1985 (8, 11)

A method to define domains of transcriptional regulating elements of a gene-of-interest typically has also involved use of phorbol esters, cyclic nucleotide analogs, concanavalin A, or steroids, molecules which are commonly known as transcriptional modulators. However, available literature shows that researchers have not considered using a transcription screen to identify specific transcriptional modulators. Apparently, success would be unlikely in doing so, however, we have demonstrated herein that this is not the case.

There is utility in developing the method of transcriptional modulation of a gene-of-interest by using such molecule as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors.

Problems associated with the therapeutic use of

recombinant biological factors include the technical difficulties of large scale protein purification, the high costs of protein production, the limited shelf-life of most proteins and in some cases a short biological half-life of the administered protein in the organism. Additionally, therapeutic delivery of proteins normally requires injection and frequently induces an immune reaction in situations where chronic administration is required. The method described herein provides a means of up-regulating the expression of proteins, e.g. membrane receptors, which are not readily amenable to administration as injectable biologicals.

Furthermore, chemical molecules specifically regulating the activity of one member of a group of closely related proteins are difficult to produce. Molecules, structurally related at the protein level, may possess distinct regulatory elements at the DNA level which control their expression. Thus, molecules such as the chemical transcriptional modulators defined herein can provide a greater opportunity for specifically modulating the activity of structurally related proteins. One example is the _ras_ oncogene family, where the H-, N- and K-_ras_ proteins are highly related but wherein the three genes have distinct structures.

Finally, the molecules described herein may also serve to mimic normal physiological response mechanisms, typically involving the coordinated expression of one or more groups of functionally related genes. Therefore, determining whether a molecule can specifically transcriptionally modulate the expression of a gene-of-interest and the ultimate clinical use of the molecule provides a therapeutic advantage over the use of single recombinant biologicals, or drugs which bind directly to the final target protein encoded by the gene-of-interest.

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## Summary of the Invention

The present invention provides a method of transcriptionally modulating the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene which is expressed by the cell.

Molecules useful in the practice of the invention are characterized as follows (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene-of-interest, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or pathological effect.
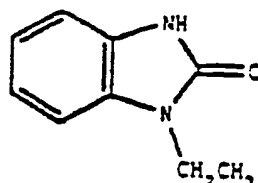
Additionally, this invention provides a method of determining whether a molecule, not previously known to be a modulator of protein biosynthesis, is capable of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene which expresses a

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polypeptide capable of producing a detectable signal, coupled to, and under the control of, the promoter, and the contacting is carried out under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable detectable signal to be produced by the polypeptide expressed by the reporter gene and the amount of the signal produced may be quantitatively determined. The amount of produced signal so determined is compared with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to thereby identify the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene-of-interest.

The present invention still further provides a method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect in the organism. The method comprises administering to the organism an amount of a molecule effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. A molecule useful in the method: (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the organism, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or
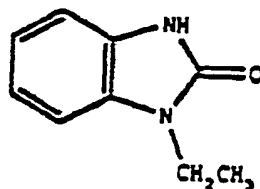
pathological effect.

This invention provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

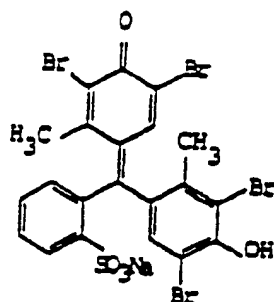effective to enhance the expression of human growth hormone by the cell.

Additionally, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone by, and thus growth of, the human being.
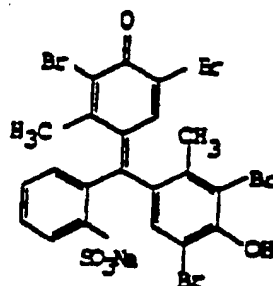
The invention also provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of

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expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:

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effective to enhance the expression of human growth hormone by the cell.

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Another provision of the present invention concerns a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:
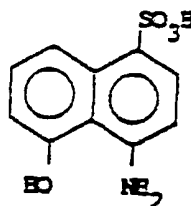
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effective to enhance expression of human growth hormone by, and thus growth of, the human being.

This invention also concerns a method for enhancing the expression of human growth hormone by a cell which (i)
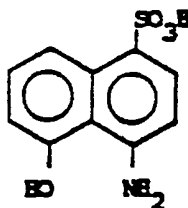
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comprises DNA encoding, and (ii) is capable of
expressing, human growth hormone, comprising contacting
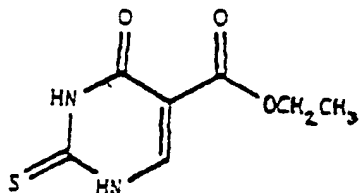the cell with an amount of a molecule having the
structure:



effective to enhance the expression of human growth
hormone by the cell.

Further, this invention provides a method of increasing
the growth of a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, human
growth hormone, comprising administering to the human
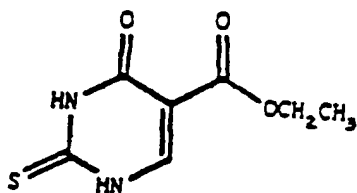being an amount of a molecule having the structure:



effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

Additionally, this invention concerns a method for
enhancing the expression of human growth hormone by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, human growth hormone, comprising
contacting the cell with an amount of a molecule having
the structure:

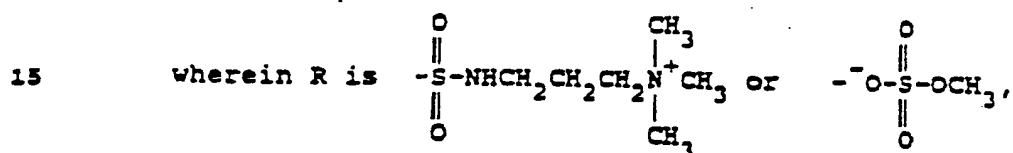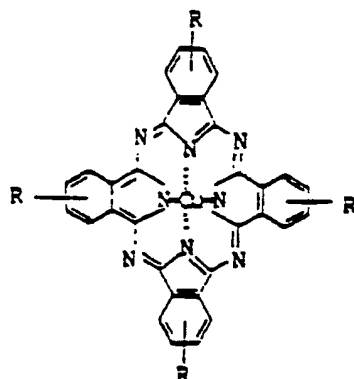effective to enhance the expression of human growth hormone by the cell.

Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of human growth hormone by, and thus growth of, the human being.
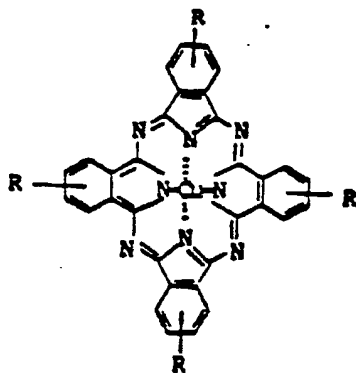
Further, the invention provides another method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

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wherein R is

$$-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-NHCH_2CH_2CH_2\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}CH_3 \quad \text{or} \quad -\overset{-}{O}-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-OCH_3,$$

effective to enhance the expression of human growth hormone by the cell.

Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:

$$\text{wherein R is } -\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-NHCH_2CH_2CH_2\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{N}}+CH_3 \text{ or } -\phantom{}^-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-OCH_3,$$

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, this invention provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:
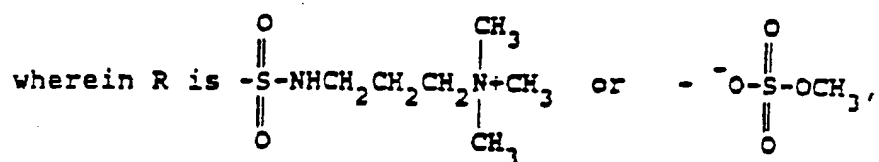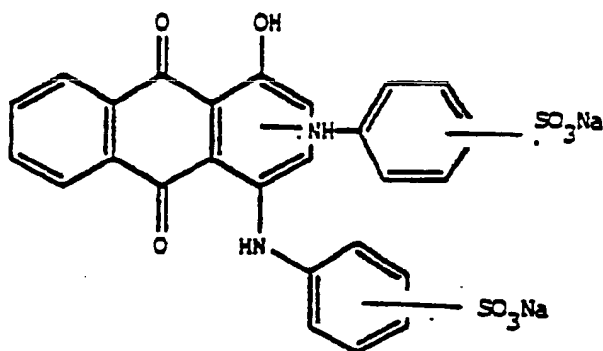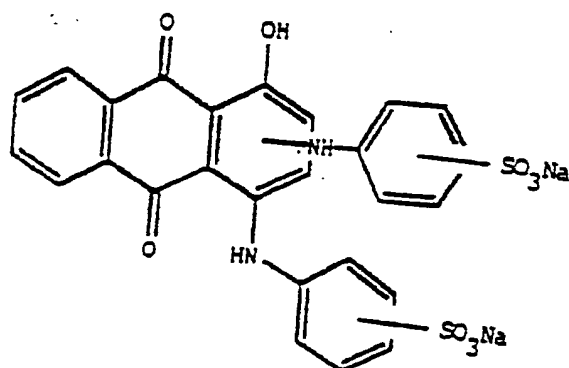


effective to enhance the expression of human growth hormone by the cell.
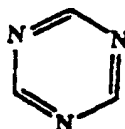
Further more, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:

$$\text{[chemical structure: anthraquinone derivative with OH, NH-phenyl-SO}_3\text{Na, and HN-phenyl-SO}_3\text{Na substituents]}$$
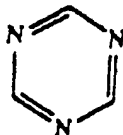
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

$$\text{[chemical structure: 1,3,5-triazine ring]}$$

effective to enhance the expression of G-CSF by the cell.

The present invention also provides a method for increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:

effective to enhance the expression of GCSF by, and
thus increase the formation of neutrophils in, the
human being.

The present invention also provides a method for
enhancing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:

effective to enhance the expression of G-CSF by the
cell.

Additionally, the invention provides a method of
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF. The method comprises
administering to the human being an amount of a
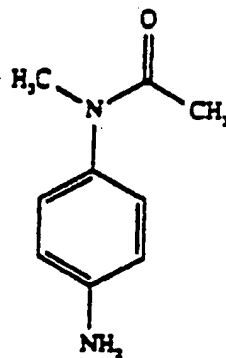molecule having the structure:

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$$
\begin{array}{c}
H_3C \diagdown \\
\quad\quad N-C \diagdown \\
\quad\quad\quad CH_3 \\
\end{array}
$$

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Further, the invention provides another method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
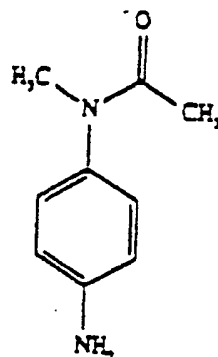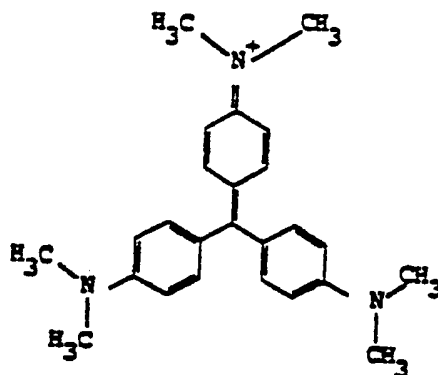
effective to enhance the expression of G-CSF by the cell.

The present invention provides another method of increasing the formation of · neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. · The method comprises administering to the human being an amount of a molecule having the structure:

$$H_3C \overset{\overset{\displaystyle CH_3}{|}}{\underset{\displaystyle +}{N}}$$

(chemical structure)

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The invention also concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF comprising contacting the cell with an amount of a molecule having the structure:

(chemical structure)

effective to enhance the expression of G-CSF by the cell.

Additionally, the invention concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

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effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.
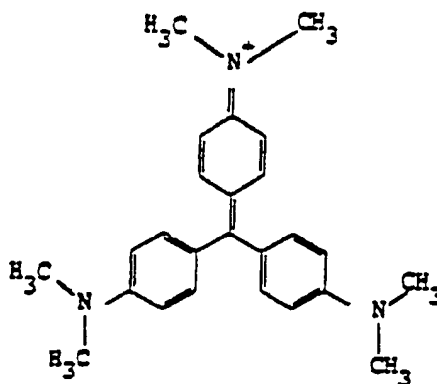
This invention further concerns a method for enhancing the expression of G-CSF by a cell-which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

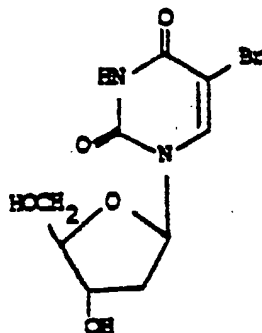

effective to enhance the expression of G-CSF by the cell.

Further the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

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effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

Additionally, the present invention concerns a method
for enhancing the expression of G-CSF by a cell which
10   (i) comprises DNA encoding, and (ii) is capable of
expressing, G-CSF, comprising contacting the cell with
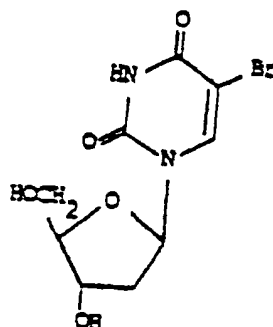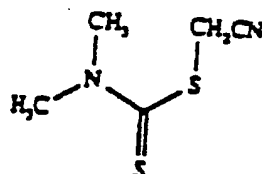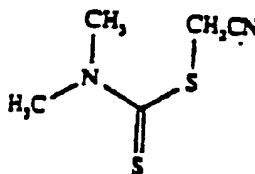an amount of a molecule having the structure:

$$NH_2$$
$$O = S = O$$

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$$CH_2NH_2$$

effective to enhance the expression of G-CSF by the
cell.

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The invention further concerns a method of increasing
the formation of neutrophils in a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing G-CSF wherein the method comprises
administering to the human being an amount of a
25   molecule having the structure:

$$
\begin{array}{c}
NH_2 \\
| \\
O = S = O \\
\end{array}
$$

(benzene ring)

$CH_2NH_2$

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Also, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

$Br$

(benzene ring)

$Cl$        $Cl$

effective to enhance the expression of G-CSF by the cell.

The invention additionally concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:
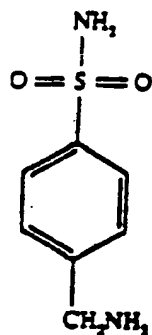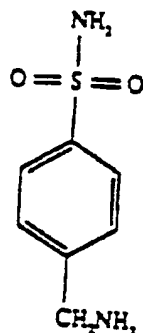
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effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Another provision of the present invention is a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

.HCl

.1/2 ZnCl2

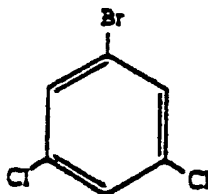effective to enhance the expression of G-CSF by the cell.

Additionally, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:

.HCl

.1/2 ZnCl2

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in the human being.

The present invention provides another method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

$$H_3C \diagdown N^+ \diagup CH_3$$

Cl⁻

Cl⁻

$$H_3C \diagdown N \diagup CH_3$$

$$CH_2CH_3$$

$$N^+ \diagup CH_3$$

$$CH_3$$

effective to enhance the expression of G-CSF by the cell.

Further, the present invention concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:

effective to enhance expression of G-CSF by, and thus
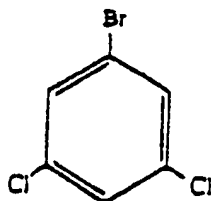increase the formation of neutrophils in, the human
being.


This invention also concerns a method of decreasing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
of a molecule having the structure:




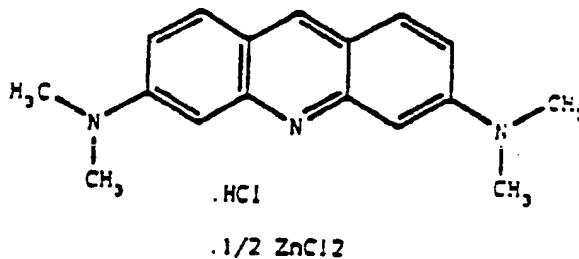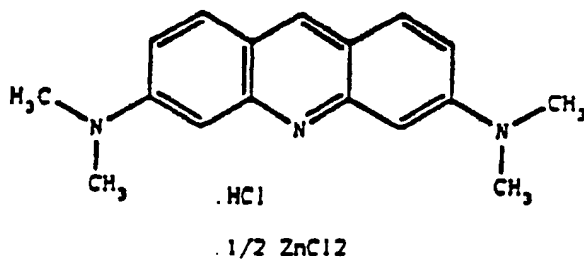effective to decrease the expression of G-CSF by the
cell.


Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises

DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

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effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

15   Further, the invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:

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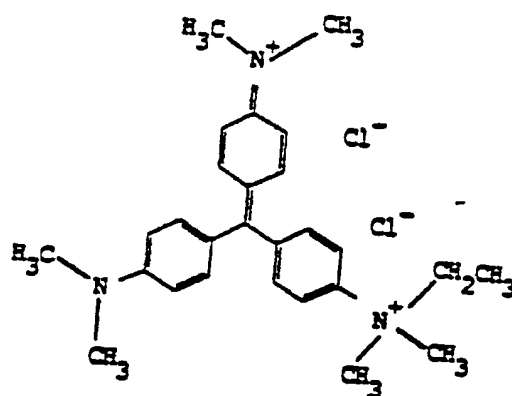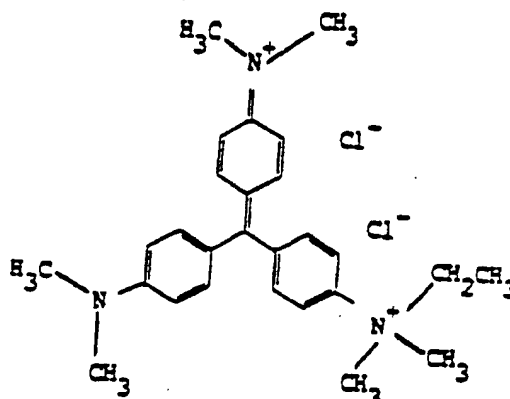effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

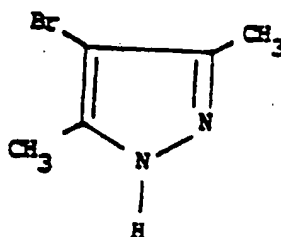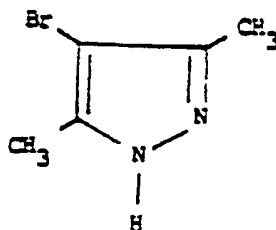effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and effecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Further, the present invention a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

$$CH_2OH$$

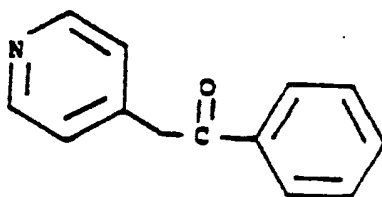effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
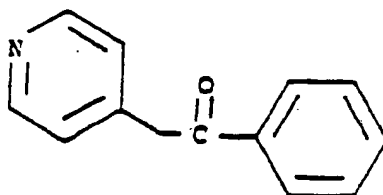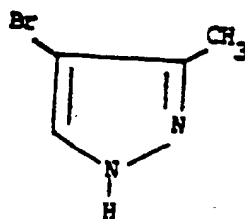of a molecule having the structure:

$$CH_2OH$$

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The present invention further provides a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of

expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:

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effective to decrease the expression of G-CSF by the
cell.

Further still the invention provides a method of
decreasing the formation of neutrophils and affecting
15      the metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

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effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic

functions of neutrophils in, the human being.

The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
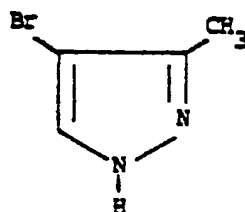


effective to decrease the expression of G-CSF by the cell.

Additionally, the present invention concerns a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
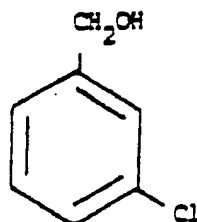
effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The present invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
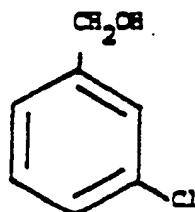cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the
cell.

Further, the invention concerns a method of decreasing
the formation of neutrophils and affecting the
metabolic functions of neutrophils, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
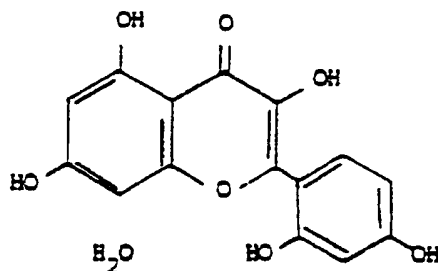of a molecule having the structure:

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
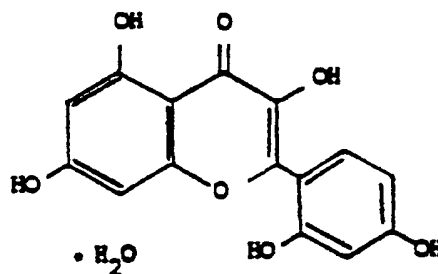functions of neutrophils in, the human being.

Moreover, the invention provides a method of decreasing
the expression of a mammary tumor virus by a cell which
(i) comprises DNA encoding, and (ii) is capable of
expressing, a mammary tumor virus. The method
comprises contacting the cell with an amount of a
molecule having the structure:

effective to decrease the expression of a mammary tumor
virus by the cell.

Finally, the invention also provides a method of
suppressing the proliferation of a mammary tumor virus
in a subject who (i) comprises DNA encoding, and (ii)
is capable of expressing, a mammary tumor virus,

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comprising administering to the subject an amount of a
molecule having the structure:

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effective to suppress the proliferation of a mammary
10      tumor virus in the subject.



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## Brief Description of the Figures

Figure 1 is a partial restriction enzyme cleavage map of the plasmid pD0432 which contains the luciferase gene from the firefly, _Photinus pyralis_.

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, _Photinus pyralis_.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, _Photinus pyralis_ and the mouse mammary tumor virus long terminal repeat.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pUXLuci which contains the luciferase gene from the firefly, _Photinus pyralis_.

Figure 5 is a partial restriction enzyme cleavage map of the plasmid phGH:CAT which contains the CAT gene and human growth hormone promoter sequences.

Figure 6 is a partial restriction enzyme cleavage map of the plasmid phGH-Luci which contains the luciferase gene from the firefly, _Photinus pyralis_ and human growth hormone promoter sequences.

Figure 7 is a partial restriction enzyme cleavage map of the plasmid pJM710 which contains G-CSF upstream sequences.

Figure 8 is a partial restriction enzyme cleavage map of the plasmid pGEM5-Luci which contains the luciferase gene from the firefly, _Photinus pyralis_.

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Figure 9 is a partial restriction enzyme cleavage map of the plasmid PG-Luc 1 which contains both the luciferase gene from the firefly, Photinus pyralis, and G-CSF upstream sequences.

Figure 10 is a bar graph illustrating induction of luciferase expression in reporter cell lines containing MMTV (M10), human growth hormone (532), and human G-CSF (G21) promoter sequences, in response to known transcriptional inducers.

Figure 11 is a bar graph illustrating the effect of steroids on luciferase expression in the MMTV reporter cell line M10.

Figure 12 is a bar graph illustrating specific induction of luciferase expression in reporter cell lines for MMTV (M10), human growth hormone (532) and human G-CSF (G21) promoters in response to chemicals identified in a high throughput screen and known transcriptional inducers.

Figure 13 is a bar graph illustrating specific inhibition of luciferase expression in reportor cell lines for MMTV (M10), human growth hormone (532), and human G-CSF (G21) in response to chemicals identified in a high throughput screen.

Figure 14 is an autoradiograph of a Northern blot illustrating increased G-CSF mRNA production by the human epithelial cell line U5637 in response to chemicals #670 and #1255 and IFN-gamma as compared to the solvent DMSO. Reprobing with beta-actin was used to normalize for the amount of mRNA that had been loaded onto the gel.

Figure 15 is an autoradiograph of a polyacrylamide gel

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illustrating an S1 nuclease protection analysis of
increased mRNA production by the human bladder carcinoma
cell line 5637 in response to lead chemicals #542, #1255,
#1793 and #1904. "RNA" indicates the sources of the RNA
preparations used in individual lanes. "Probe" indicates
the mRNA-specificities of probes used in individual
lanes. "Compound" lists the compounds with which the 5637
cells were treated prior to RNA extraction and loading on
individual gel lanes ("Cyclo" means cycloheximide).
"Conc" indicates three different compound concentrations
used in the experiment (L = low, M = medium, H = high).
G, GM and A indicate the correct sizes of G-CSF-, GM-CSF-
and Actin-specific nuclease-protected mRNA/Probe hybrids.

Figure 16 illustrates a dose response analysis of
chemicals #80, #670, and #1780 using the G-CSF reporter
cell line G21. The amount of luciferase expression is
indicated in arbitrary units.

Figure 17 is a bar graph illustrating increased G-CSF
secretion by 5637 cells treated for 48 hours in serum-
containing media with the samples indicated on the
abscissa. TNF-alpha was used at 5 ng/ml. Chemicals #542
and #1780 were used at 50 uM or 1 uM and 0.2 uM final
concentration, respectively. Both chemicals were used in
DMSO at a final concentration of 0.5 %. The ordinate
indicates the concentration of G-CSF secreted into 5 ml
of serum-containing media by 25 square cm of confluent
5637 cells.

Figure 18 illustrates a dose response analysis of
chemical #542 using the G-CSF reporter cell line G 21
(solid line) and the MTT respiratory inhibition
cytotoxicity assay (dotted line). Respiratory inhibition
in percent of untreated control cells (Ordinate, left

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scale) and luciferase expression of #542- treated over
solvent-treated cells (ordinate, right scale) are plotted
against #542 concentration (abscissa).

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## Detailed Description of the Invention

The present invention provides a method of
transcriptionally modulating the expression of a
homologous gene-of-interest, the expression of which is
associated with a defined physiological or pathological
effect within a multicellular organism. The method
comprises contacting a cell which is capable of
expressing the gene with an amount of a molecule
effective to transcriptionally modulate the expression of
the gene. Modulating the expression of the gene affects
the level of the protein encoded by the gene which is
expressed by the cell. As used herein a homologous gene-
of-interest means a gene which is naturally associated
with an organism or virus encoding and expressing the
gene. Therefore, this definition would not include a
synthetic or recombinant gene constructed by genetic
engineering methods so as to position the gene-of-
interest under the control of a heterologous promoter.

The term "physiological effect" as used herein is defined
as an effect characteristic of, or appropriate to, an
organism's healthy or normal functioning. Further, the
term "pathological effect" as used herein is defined as
an effect altered or caused by disease.

Molecules useful in the practice of the invention are
characterized as follows (a) do not naturally occur in
the cell, (b) specifically transcriptionally modulate
expression of the gene-of-interest, and (c) bind to DNA
or RNA or bind to a protein through a domain of such
protein which is not a ligand binding domain of a
receptor which naturally occurs in the cell, the binding
of a ligand to which ligand binding domain is normally

associated with the defined physiological or pathological
effect.

In one example of this invention, the molecule would not
occur naturally in any cell of a higher eucaryotic
organism.   In another example of this invention the
molecule would not occur naturally in any cell, e.g. a
mineral.  In yet another example, the molecule would not
occur naturally, e.g. a synthetic molecule.

The phrase "specifically transcriptionally modulate
expression of the gene-of-interest" as used herein means
modulating the    expression of the gene-of-interest
without modulating the expression of other genes in the
cell in a way which would cause an adverse effect on (a)
an organism containing the cell in the case where the
cell is within the organism or (b) the growth or the
culturing of the cell, in the case where the cell is
being grown or cultured to make a product where the
amount of product produced is associated with expression
of the gene-of-interest. However, within this definition
where the drug is used to treat, for example, parasitic
infection, drug application is intended to cause an
adverse effect on the cells of the parasite (which may
contain the gene-of-interest), but not on the cells of
the host organism.   In this context, a gene-of-interest
may constitute a single gene or a limited number of genes
whose expression can be functionally coordinated.  One
example of coordinate gene regulation is exhibited by the
physiological growth modulators termed the transforming
growth factor-$\beta$ family of polypeptides (TGF-$\beta$s) (90).
TGF-$\beta$s control the extracellular matrix (ECM) by; (1)
increasing expression of the genes encoding ECM
polypeptides, e.g. collagen, fibronectin and osteopontin;
(2) increasing expression of receptors for the ECM, e.g.

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the integrins; and (3) increasing expression of protease inhibitors (e.g. TIMP and PAI-I) while decreasing expression of secreted proteases (e.g. collagenase and stromelysin). This coordinate regulation may allow TGF-βs to be useful in the repair of surface wounds, cartilage and bone. Molecules with properties as described in this invention, with lower molecular weights than TGF-β, and which can be either chemically synthesized or readily isolated from natural sources, yet mimic the coordinate regulation of extracellular matrix as induced by the TGF-βs, have significant advantages as therapeutic agents over the use of such complex polypeptides.

Moreover, the phrase "transcriptionally modulate the gene-of-interest" infers a notion of directness. Thus, as used herein, "transcriptionally modulate expression of a gene-of-interest" by a molecule means the effect upon transcription of the gene resulting from either (a) direct binding of the molecule to DNA or RNA, a DNA- or RNA-binding protein, and/or a DNA- or RNA- binding protein complex, or (b) direct binding of the molecule to a protein which directly chemically modifies a DNA- or RNA- binding protein or protein complex.

As used herein "chemically modifies" a DNA- or RNA-binding protein or protein complex means to modify the protein or protein complex through a chemical reaction, including but not limited to, phosphorylation, glycosylation, methylation, acetylation, adenoribosylation, acylation, myristylation, reduction, oxidation, covalent oligomerization or polymerization or proteolytic cleavage.

The invention provides a cell capable of expressing the

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gene-of-interest, the expression of which is associated
with a defined physiological or pathological effect
within a multicellular organism. The cell may be a human
cell, an animal cell, a plant cell or any eucaryotic cell
or procaryotic cell from whatever source.

Further, in the practice of the invention, the gene-of-
interest whose expression is associated with a defined
physiological or pathological effect within a
multicellular organism, may be a human gene.

Moreover, the gene-of-interest may encode a hematopoietic
protein. Hematopoietic proteins may include, but are not
limited to, colony stimulating factors and erythropoietin
(EPO).

Examples of colony stimulating factors useful in the
practice of this invention include, but are not limited
to, granulocyte-macrophage colony stimulating factor
(GM-CSF), granulocyte colony stimulating factor (G-CSF),
and macrophage colony stimulating factor (M-CSF).

Further, the gene-of-interest of the invention may encode
an interleukin (IL) or a cytokine, or a growth modulating
factor. One example of such a growth modulating factor
would be a member of the transforming growth factor-ß
(TGF-ß) family i.e. TGF-ß1 or TGF-ß2 or TGF-ß3. A gene-
of-interest may also encode a receptor for a steroid
hormone, such as the testosterone receptor or the
estrogen receptor or a receptor for a TGF-ß.

The gene-of-interest may also encode a growth hormone.
Examples of growth hormones include, but are not limited
to, human, bovine, porcine, avian, ovine, piscine, and

equine growth hormones. Additionally, the gene-of-interest may also encode polypeptide analogs of the above-identified growth hormones. Additionally, a gene-of-interest may encode a growth hormone releasing factor.

The present invention also provides a viral gene as the gene-of-interest. The viral gene may be a retroviral gene. Retroviral genes of the invention may be from the HIV, HTLV-1, or HTLV-2 virus.

In the practice of the invention the viral gene may be a gene from a hepatitis virus, a herpes virus, a papilloma virus, a cytomegalovirus, or an animal virus.

Animal viruses of the invention may include, but are not limited to, pseudorabies, Marek's, Newcastle's Disease, and IBR viruses.

The gene-of-interest, whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may also be a plant gene. The plant gene may encode an agronomically important trait. Examples of agronomically important traits may include, but are not limited to, germination, sprouting, flowering, fruit ripening, salt tolerance, herbicide resistance, pesticide resistance, fungicide resistance, temperature resistance, and growth.

Additionally, in the practice of the invention the gene-of-interest may be a protozoan gene. Examples of protozoans may include, but are not limited to, a selection from the group consisting of _Trypanosoma_, _Plasmodium_, _Leishmania_, _Giardia_, _Entamoeba_, _Toxoplasma_, _Babesia_, and _Cryptosporidiosis_.

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Moreover, the gene-of-interest whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may be a helminth gene.

Further, the gene-of-interest may also be an oncogene. Examples of oncogenes may include, but are not limited to, the phl-abl oncogene, the neu oncogene, or the src oncogene. Additionally, the oncogene may be selected from the group consisting of H-ras, N-ras, and K-ras oncogenes.

The present invention additionally provides that the gene-of-interest, whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may encode a naturally occurring receptor. The naturally occurring receptor may be the human low density lipoprotein (LDL) receptor. Further, the receptor may be the receptor for a hemopoietic protein. Examples of hematopoietic proteins may include, but are not limited to, a selection from the group consisting of M-CSF, G-CSF, GM-CSF, and EPO.

The naturally occurring receptor encoded by the gene-of-interest may also be the receptor for an interleukin (IL). Examples of an IL may include, but are not limited to, a selection from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 and IL-8.

Additionally, in the practice of the invention, the naturally occurring receptor may be a cell surface protein which mediates infection of the cell by a virus. Examples of viruses may include, but are not limited to, HIV, HTLV-1, HTLV-2, a hepatitis virus, a herpes virus, a papilloma virus, a cytomegalovirus and a rhinovirus.

In one example of the invention, the receptor which naturally occurs in the cell is a testosterone receptor. In another example of the invention, the receptor which naturally occurs in the cell is an estrogen receptor.

Typically, a ligand, in the context of this invention, is a molecule with a molecular weight of less than 5,000 daltons, more typically less than 2,000 daltons.

This invention also provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene which expresses a polypeptide capable of producing a detectable signal, coupled to, and under the control of, the promoter. The polypeptide expressed by the reporter gene produces the detectable signal under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable detectable signal to be produced by the polypeptide expressed by the reporter gene.

Quantitatively determining the amount of the signal produced requires comparing the amount of signal produced compared to the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule. The

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comparison permits the identification of the molecule as
one which causes a change in the detectable signal
produced by the polypeptide expressed by the reporter
gene and thus identifying the molecule as a molecule
5       capable of transcriptionally modulating the expression of
the gene-of-interest.

The phrase "a modulatable transcriptional regulatory
sequence of a gene-of-interest" as used herein concerns
10      a DNA sequence capable of regulating the initiation of
transcription from the promoter of the gene-of-interest.

Molecules useful in the practice of this invention have
15      the following characteristics.  The molecule does not
naturally occur in the cell. The molecule specifically
transcriptionally modulates expression of the gene-of-
interest. Further, the molecule binds to DNA or RNA or
binds to a protein through a domain of such protein which
20      is not a ligand binding domain of a receptor which
naturally occurs in the cell. The binding of a ligand to
the ligand binding domain is normally associated with the
defined physiological or pathological effect.

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The term "promoter" of a gene-of-interest is defined
herein as  a minimal DNA sequence necessary for specific
initiation of transcription.

30      In the practice of the invention the sample may comprise
cells in monolayers or cells in suspension. The cells of
the invention may comprise human, animal, or plant cells.
In one example of the invention, the cells are bacterial
cells.  In another example of the invention, the cells
35      are fungal cells.

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Further, the invention provides that the predefined number of cells contained in the sample may be from about $2 \times 10^2$ to about $5 \times 10^5$ cells, typically from about $10^3$ to about $5 \times 10^4$ cells. In theory, the ideal method might use only one cell. In practice, the method may include at least 10 cells per samples. More practically the method may include at least 100 cells per sample.

The invention also provides that the predetermined amount of the molecule to be tested may be based on the volume of the sample. Further, the predetermined amount of the molecule to be tested may be from about 1pM to about 500$\mu$M. Typically, in a primary highthroughtput screen the predetermined amount would be from about 10nM to about 500$\mu$M. Typically, in a secondary screen to evaluate initial lead compounds, the predetermined amount would be from about 1pM to about 20 $\mu$M.

Further, the invention provides that contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested may be effected from about 1 to about 24 hours, typically from about 2 to about 12 hours. Moreover, contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested may be effected with more than one predetermined amount of the molecule to be tested. The molecule to be tested may be, but is not limited to, a purified molecule. As used herein, the molecule may also be a mixture of different molecules, e.g. in a fermentation broth or in a natural product extract. Typically, a fermentation broth would be produced by fermentation of a suitable sample under standard conditions known to those skilled in the art. Examples of natural product extracts would include, but are not restricted to, plant extracts, seaweed extracts,

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insect extracts, spider venoms, etc.

A minimal modulatable transcriptional regulatory sequence
may comprise a few nucleotides in length, such as the so-
called CCAAT box motif, the octamer binding motif or the
heat shock element (91). A gene-of-interest may typically
possess multiple motifs with different combinations of
modulatable transcriptional regulatory sequences. It has
been shown that enhancer elements or other regulatory
motifs can be present at many kilobases upstream or
downstream from the transcription start site of a gene-
of-interest. Thus, as. used herein, a modulatable
transcriptional regulatory sequence may comprise a few
nucleotides or may be naturally or synthetically
constructed having multiple elements spanning several
hundred kilobases in length. Transcription can also be
influenced by the particular nuclear location or local
chromatin configuration. In order to optimize a screen
for substances that modulate regulatory elements, it may
be preferable to situate a reporter gene at the exact
chromosomal region where the gene-of-interest resides
normally. However, it would be clear to one skilled in
the art that the reporter gene may be situated anywhere
in a chromosome. In one example in the invention, the
modulatable transcriptional regulatory sequence comprises
a cloned genomic regulatory sequence. In another example
of the invention, the DNA consists essentially of more
than one modulatable transcriptional regulatory sequence.

A series of experiments described by K.R. Thomas and M.R.
Capecchi (44,45,46) have shown that it is possible to
direct transfected or microinjected DNA, using homologous
DNA sequences, to a specific chromosomal location in
mammalian cells. Using homologous recombination, it is
possible to replace a gene-of-interest with other

sequences at a high frequency. The materials required include a reporter gene (such as luciferase), a selectable marker (such as the neomycin phosphotransferase II gene from Tn5), and sequences derived from the gene-of-interest. These materials would be used to construct a vector which would include, in one embodiment of this invention, a fusion consisting of the gene-of-interest (free of its normal upstream promoter sequences) and the reporter gene where the reporter gene product is still active.

Two types of selectable markers, one positive and one negative, such as the Herpes Simplex thymidine kinase (TK), greatly enhance the likelihood of achieving homologous recombination. Use of one copy of the TK gene located outside the homologous regions at either end allows for selection of "correct" homologous recombinants. Neomycin resistant cells which are recombined randomly will usually insert via the ends of the linearized, transfected DNA and thus include the TK gene(s). This will allow for selection against these cells in the presence of gancyclovir which is converted to a toxic product. This is the method specifically described by Capecchi. However, this method has not been used to produce cell lines to screen for compounds which modulate the expression of a target gene-of-interest as described herein.

The invention provides that the reporter gene in the DNA contained in the cell sample, which expresses a polypeptide capable of producing a detectable signal coupled to, and under control of, the promoter, may be inserted downstream of the endogenous promoter of the gene-of-interest by homologous recombination. The following provides a method of determining whether a

molecule not previously known to be a modulator of
protein biosynthesis is capable of transcriptionally
modulating the expression of a gene-of-interest which
comprises contacting a sample which contains a predefined
number of cells with a predetermined amount of a molecule
to be tested, each such cell comprising DNA consisting
essentially of (i) a modulatable transcriptional
regulatory sequence of the gene-of-interest, (ii) a
promoter of the gene-of-interest, and (iii) a DNA
sequence transcribable into mRNA coupled to and under the
control of, the promoter, under conditions such that the
molecule, if capable of acting as a transcriptional
modulator of the gene-of-interest, causes a measurable
difference in the amount of mRNA transcribed from the DNA
sequence, quantitatively determining the amount of the
mRNA produced, comparing the amount so determined with
the amount of mRNA detected in the absence of any
molecule being tested or upon contacting the sample with
any other molecule, and thereby identifying the molecule
as one which causes a change in the amount of detectable
mRNA, and thus identifying the molecule as a molecule
capable of transcriptionally modulating the expression of
the gene-of-interest.  In one example of the above-
described method, the molecule (a) does not naturally
occur in the cell, (b) specifically transcriptionally
modulates expression of the gene-of-interest, and (c)
binds to DNA or RNA or binds to a protein through a
domain of such protein which is not a ligand binding
domain of a receptor which naturally occurs in the cell,
the binding of a  ligand to which ligand binding domain
is normally associated with a defined physiological or
pathological effect.


Modulatable transcriptional regulatory sequences may also

occur within introns of a gene-of-interest. Typically,
the exact location of all such regulatory sequences of a
gene-of-interest will be unknown. In one example of this
invention, the reporter gene will be a reporter gene
which can be transcribed into mRNA which can be
quantified as a detectable signal. This approach would
allow (i) allow all the modulatable transcriptional
regulatory sequences of the gene to be in their native
environment and arrangement relative to the gene-of-
interest including any and all transcriptional regulatory
sequences within introns, (ii) obviate the necessity of
using permanent cell lines to construct reporter cell
lines and (iii) not require knowledge of promoter and
modulatable transcriptional regulatory sequences.

In one example of the invention, mRNA is detected by
quantitative polymerase chain reaction.   Methods for
direct detection of mRNA in a rapid and quantitative
manner that fulfill the needs of a high throughput
screening method described in this invention are not
available. A suitable assay would have to meet the
following criteria: (i) specific detection of fewer than
50,000 mRNA molecules, (ii) short assay time of a few
hours, (iii) simple chemistry, that is amenable to
automation. Ideally a "single tube" assay would be used
which can be carried out within the same container in
which the cells to be tested have been cultured, by
eliminating transfer steps which may be time consuming
and contribute to assay variation.

Several techniques have been described by others, which
may be modified to be useful in the context of this
invention. All assays are based on the principle of using
a single stranded nucleotide probe containing sequences
complementary to the target mRNA transcribed from the

gene-of-interest which probe is attached to a solid support to rapidly concentrate (capture) the mRNA out of a crude cell lysate. Cell lysates can be prepared such that ribonucleases are inhibited while hybridization of nucleic acids remains possible, for example in high concentrations of guanidinium isothiocyanate (47). Target mRNA bound to the solid phase can be freed of contaminants by intensive washing and subsequently detected with a second probe which is complementary to a different region of the target mRNA than the capture probe. The second probe would carry an appropriate detectable label.

To obtain optimal sensitivity i.e. obtain a maximal number of labels being specifically bound to a target molecule in the presence of a minimal number of labels being unspecifically retained by other components of the assay mixture, various techniques can be applied to increase signal to noise ratios. A procedure termed reversible target capture has been designed to reduce the background noise by capturing the target molecule - label complexes onto capture probes linked to solid phase, release of the complexes and transfer of the complexes onto new capture probes linked to solid phase and so forth, cycling between steps. Background is reduced by this procedure, because the release step is designed not to release non-specifically bound label that will not therefore be transferred together with the target molecules and will eventually be removed from the mixture (48). This method has a reported sensitivity of 15,000 target mRNA molecules, it has, however the disadvantage of involving multiple transfer steps into new containers.

Beside reducing background the signal to noise ratio can be improved by binding more than one label to a target

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molecule or by creating many copies of an initial target molecule (amplification). For example, DNA may be detected by a method using an amplification oligonucleotide with a branched structure that can specifically bind to the target molecule and at the same time to many molecules carrying label resulting in an up to 500-fold amplification of specific signal. This method can potentially be carried out in a single tube. Its reported sensitivity is 60,000 target DNA molecules with an assay duration of 4 hours (48). Further methods of amplification include the hybridization dependant replication of RNA via the Q-β bacteriophage system (49) or polymerase chain reaction based replication of a cDNA copy of the target mRNA molecule (50). The methods described herein above, have not been used by others in the context of this invention. However, in light of the subject invention, it would be clear to those skilled in the art that use of these methods would facilitate the practice of this invention.

The invention also provides the use of a reporter gene whose product is easily detectable. The reporter gene may encode a luciferase, chloramphenicol acetyltransferase, β glucuronidase, β galactosidase, neomycin phosphotransferase, or guanine xanthine phosphoribosyltransferase.

The present invention also provides a screening method for determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest which comprises separately contacting each of a plurality of substantially identical molecules, e.g. more than about $10^6$ samples, preferably more than about

$10^6$ samples.

In theory, the ideal screening method provides contacting one sample with a predetermined amount of each molecule to be tested. In practice, the method may include contacting at least 3 substantially identical samples, with a predetermined amount of each molecule to be tested, more practically contacting about 9 substantially identical samples with a predetermined amount of each molecule to be tested.

Moreover, the invention provides a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes of interest in a panel of such genes. The method comprises essentially simultaneously screening the molecules against each of the genes of interest by separately contacting each of a plurality of substantially identical samples, each of which contains a predefined number of cells, with a predetermined amount of a different molecule to be tested.

The screening method described hereinabove also provides a quantitative testing range wherein at least about $10^3$ samples per week are contacted with different molecules against either one or a panel of such genes of interest.

Further, in the practice of this invention a method for determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest is provided. This method works in human and in animal cells. Data is presented so that one skilled in the art could make the method work in fungal cells. In

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addition, one skilled in the art would realize that the method may be effected in plant cells or in bacterial cells. In the case wherein luciferase is used as a reporter, this enzyme has been shown by others to be active in plant cells (89).

The invention also provides a method for transcriptionally modulating in a multicellular organism, the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect in the organism. The method comprises administering, e.g. oral administration, administration as a suppository, topical contact, intravenous, intramuscular or subcutaneous administration, to the organism an amount of a molecule effective to transcriptionally modulate expression of the gene thereby affecting the defined physiological or pathological effect. The molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the organism. Moreover, the binding of a ligand to the ligand binding domain is normally associated with the defined physiological or pathological effect.

In the practice of the invention, examples of a multicellular organism include, but are not limited to, a human, an animal, or a plant.

The defined pathological effect may be associated with a disorder and the modulated expression of the gene-of-interest may be associated with amelioration of the disorder. Further, examples of disorders include but are

not limited to, a selection from the group consisting of
cancer, a hematopoietic dysfunction, diabetes, tissue
inflammation, atherosclerosis, dysfunctions of memory or
learning, dysfunctions in a cholesterol or other
5       metabolic pathway; viral, fungal or parasitic infections.
Thus, the gene-of-interest is not necessarily part of the
normal genetic make-up of the multicellular organism but
rather can be introduced via infection by a pathogen. In
one example of the invention, the defined physiological
10      effect is growth and the organism is an animal such as a
man, a cow, a pig, a bird, a fish, a sheep or a horse.
In another example of the invention the defined
physiological or pathological effect is an agronomically
important trait. In a further example of the invention
15      administration comprises topical contact. Further in
another example of the invention, administration
comprises oral, transdermal, intravenous, intramuscular
or subcutaneous administration. Further in one example
of the invention, provides the gene-of-interest encodes
20      a naturally occurring receptor. Further, in one example
of the invention, the receptor is a testosterone
receptor. Further, in another example of the invention
the receptor is an estrogen receptor. Further, in
another example of the invention, the receptor which
25      naturally occurs in the cell is a testosterone receptor.
Further, in another example of the invention the receptor
which naturally occurs in the cell is an estrogen
receptor. Further, in another example of the invention
the gene-of-interest encodes a TGF-ß receptor. Further,
30      in another example of the invention the TGF-ß is TGF-ß1.
Further, in another example of the invention the TGF-ß is
TGF-ß2. Further, in another example of the invention the
TGF-ß is TGF-ß3. Further, in another example of the
invention the gene-of-interest encodes an oncogene.
35      Further, in another example of the invention the oncogene

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is the _neu_ oncogene. Further, in another example of the invention the oncogene is selected from the group consisting of H-, N-, and K-_ras_ oncogenes.

5

Additionally, the method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest provides that growth may be the defined physiological effect and the organism is an animal such as a cow, a pig, a bird, a fish, a sheep, or a horse.
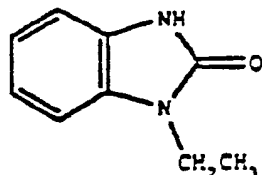
10

Further, the method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest provides that the agronomically important trait may be the defined physiological or pathological effect.

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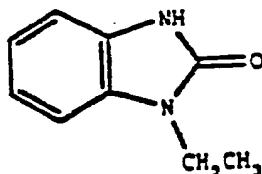Additionally, the invention provides a method for enhancing the

expression of human growth hormone by a

cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:
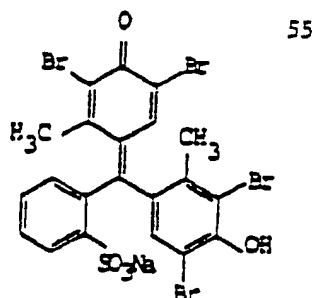


effective to enhance the expression of human growth hormone by the cell.

Additionally, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:
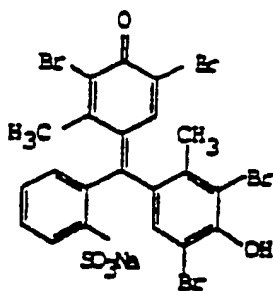


effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention also provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:

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effective to enhance the expression of human growth hormone by the cell.

Another method of the invention provides increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

This invention also concerns a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:
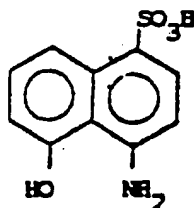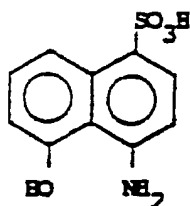
effective to enhance the expression of human growth
hormone by the cell.

Further, this invention provides a method of increasing
the growth of a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, human
growth hormone, comprising administering to the human
being an amount of a molecule having the structure:

effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

Additionally, this invention concerns a method for
enhancing the expression of human growth hormone by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, human growth hormone, comprising
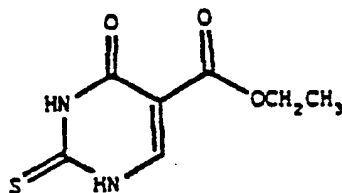contacting the cell with an amount of a molecule having
the structure:

effective to enhance the expression of human growth
hormone by the cell.

The present invention further provides a method of
increasing the growth of a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing, human growth hormone. The method comprises
administering to the human being an amount of a
molecule having the structure:

effective to enhance expression of human growth hormone
by, and thus growth of, the human being.

The invention provides another method for enhancing the
expression of human growth hormone by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, human growth hormone. The method comprises
contacting the cell with an amount of a molecule having
the structure:

wherein R is $-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-NHCH_2CH_2CH_2\overset{CH_3}{\underset{CH_3}{\overset{|}{N}}}^+CH_3$ or $-\ ^-O-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-OCH_3,$

effective to enhance the expression of human growth hormone by the cell.

Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:
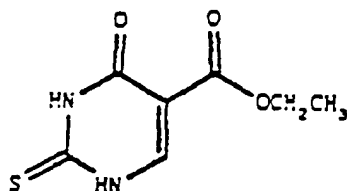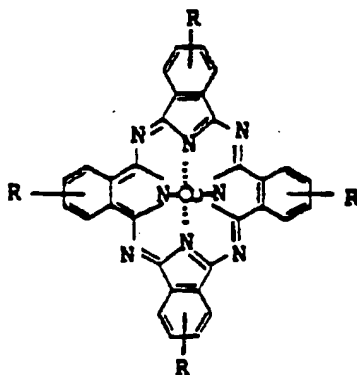


wherein R is $-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-NHCH_2CH_2CH_2\overset{CH_3}{\underset{CH_3}{\overset{|}{N}}}^+CH_3$ or $-\ ^-O-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-OCH_3,$

effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, another method of the invention provides for
enhancing the expression of human growth hormone by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, human growth hormone.   The
method comprises contacting the cell with an amount of
a molecule having the structure:



effective to enhance the expression of human growth
hormone by the cell.

The invention provides yet another method of increasing
the growth of a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, human
growth hormone.   The method comprises administering to
the human being an amount of a molecule having the
structure:

effective to enhance expression of human growth hormone
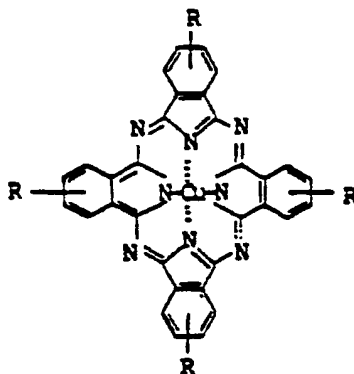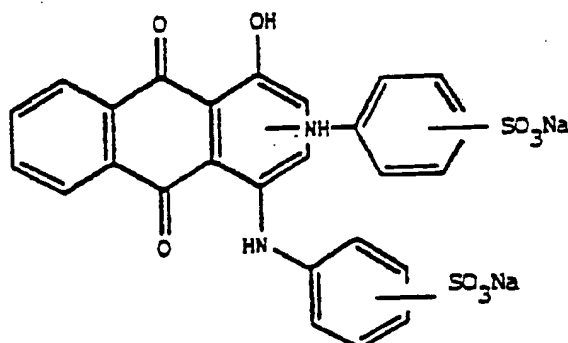by, and thus growth of, the human being.

The present invention provides a method for enhancing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
of a molecule having the structure:

effective to enhance the expression of G-CSF by the
cell.

The present invention also provides a method for
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF. The method comprises
administering to the human being an amount of a
molecule having the structure:

effective to enhance the expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

5      The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

10

15     effective to enhance the expression of G-CSF by the cell.

Additionally, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is
20     capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:

25

effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in the human
being:

A method of the invention involves enhancing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
of a molecule having the structure:



effective to enhance the expression of G-CSF by the
cell.

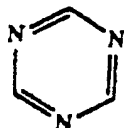Still another method of the invention involves
increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF. The method comprises
administering to the human being an amount of a
molecule having the structure:

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effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

5       The present invention provides a method for enhancing
        the expression of G-CSF by a cell which (i) comprises
        DNA encoding, and (ii) is capable of expressing, G-CSF,
        comprising contacting the cell with an amount of a
        molecule having the structure:

10



15      effective to enhance the expression of G-CSF by the
        cell.

        Additionally, the invention concerns a method of
        increasing the formation of neutrophils in a human
        being who (i) comprises DNA encoding, and (ii) is
        capable of expressing, G-CSF, comprising administering
20      to the human being an amount of a molecule having the
        structure:



25

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effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

This invention further concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
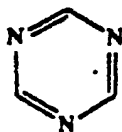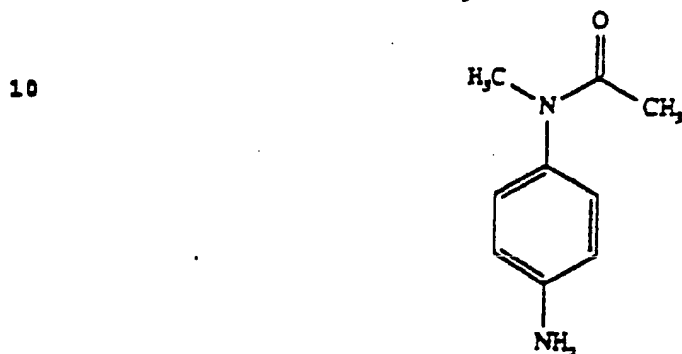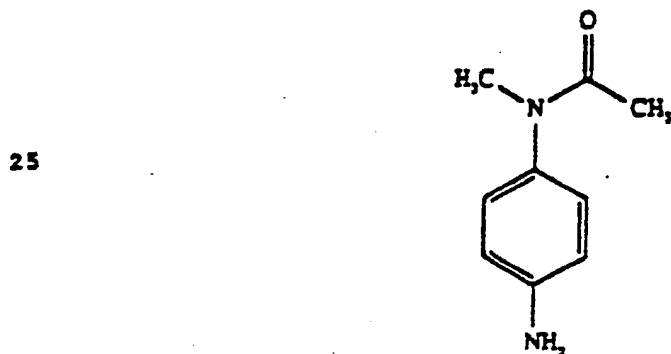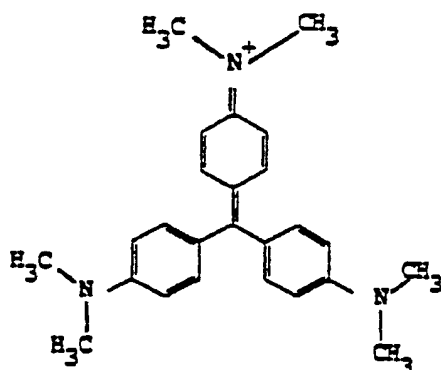
$$\underset{\text{S}}{\overset{\text{CH}_3 \quad\quad CH_2CN}{\underset{\underset{H_3C}{|}}{N}}}$$

effective to enhance the expression of G-CSF by the cell.

Further provided is a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:

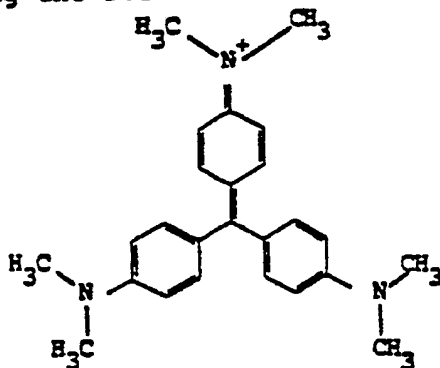$$\underset{\text{S}}{\overset{\text{CH}_3 \quad\quad CH_2CN}{\underset{\underset{H_3C}{|}}{N}}}$$

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Additionally, the present invention concerns a method
for enhancing the expression of G-CSF by a cell which
(i) comprises DNA encoding, and (ii) is capable of
expressing, G-CSF, comprising contacting the cell with
5    an amount of a molecule having the structure:

$$
\begin{array}{c}
NH_2 \\
| \\
O = S = O \\
\end{array}
$$

10

effective to enhance the expression of G-CSF by the
15   cell.

Also, the invention provides a method of increasing the
formation of neutrophils in a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing G-CSF, wherein the method comprises
20   administering to the human being an amount of a
molecule having the structure:

$$
\begin{array}{c}
NH_2 \\
| \\
O = S = O \\
\end{array}
$$

25

effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Further, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



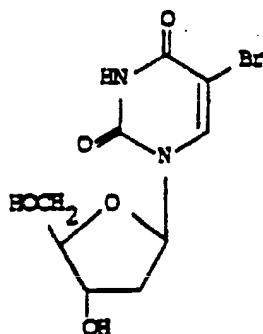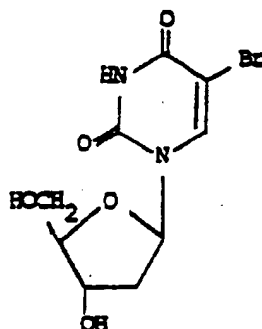effective to enhance the expression of G-CSF by the cell.

The invention additionally concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:
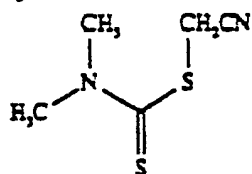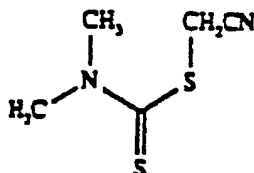


effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The invention provides a method for enhancing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing G-CSF. The
method comprises contacting the cell with an amount of
a molecule having the structure:



.HCl

.1/2 ZnCl2

effective to enhance the expression of G-CSF by the
cell.

The invention further provides a method of increasing
the formation of neutrophils in a human being who (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises administering
to the human being an amount of a molecule having the
structure:



.HCl

.1/2 ZnCl2

effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in the human
being.

Additionally, the invention provides a method for
enhancing the expression of G-CSF by a cell which (i)
5    comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:



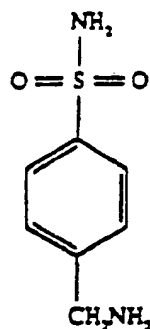effective to enhance the expression of G-CSF by the
cell.

Further, the present invention provides a method of
20   increasing the formation of neutrophils in a human
being who (i) comprises DNA encoding, and (ii) is
capable of expressing G-CSF. The method comprises
administering to the human being an amount of a
molecule having the structure:

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effective to enhance expression of G-CSF by, and thus
increase the formation of neutrophils in, the human
being.

This invention also concerns a method of decreasing the
expression of G-CSF by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
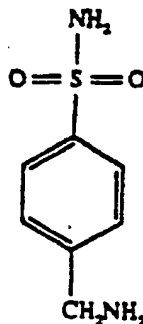of a molecule having the structure:



effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as

superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
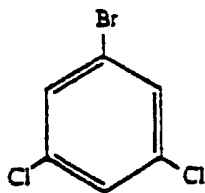


effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Further, the invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:
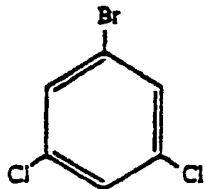
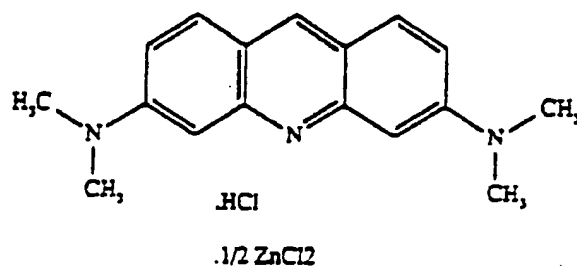effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as
superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:



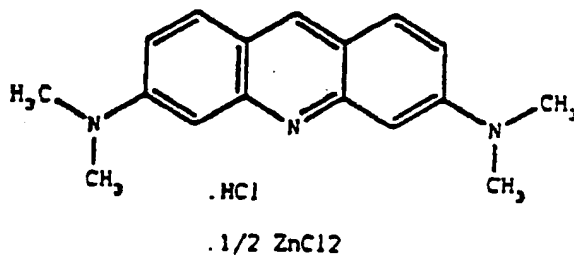effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The invention further provides a method of decreasing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.
The method comprises contacting the cell with an amount
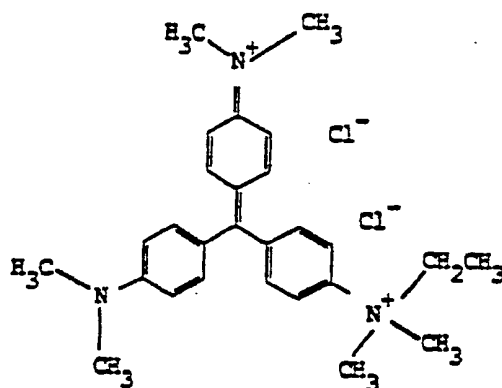of a molecule having the structure:

72



effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as
superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

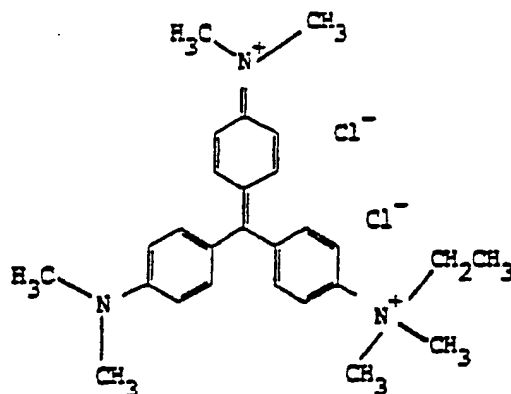Further, the present invention a method of decreasing
the expression of G-CSF by a cell which (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF.

The method comprises contacting the cell with an amount
of a molecule having the structure:

5



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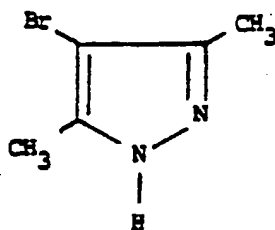effective to decrease the expression of G-CSF by the
cell.

Additionally, this invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as
superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

20



25

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

The present invention further provides a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the
cell.

Further still the invention provides a method of
decreasing the formation of neutrophils and affecting
the metabolic functions of neutrophils, such as
superoxide or peroxide formation, which has
implications for the treatment of inflammatory and
autoimmune disorders in a human being who (i) comprises
DNA encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an amount
of a molecule having the structure:

75

OH    O
        OH



HO
              O

    · H₂O        HO        OH

effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.
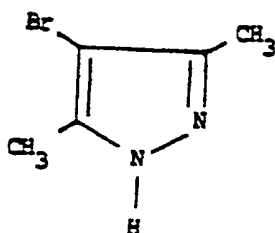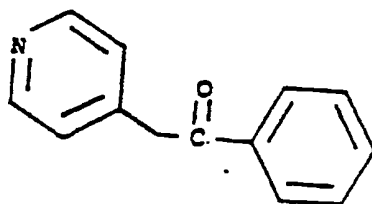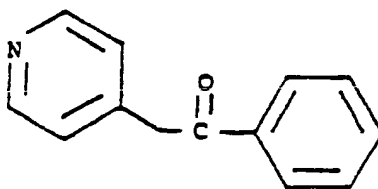
The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:

CONH₂
        OH



effective to decrease the expression of G-CSF by the cell.

Additionally, the present invention concerns a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF,

comprising administering to the human being an amount
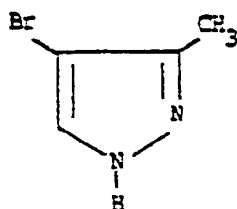of a molecule having the structure:

5



10

effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

15   The present invention also concerns a method of
decreasing the expression of G-CSF by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, G-CSF. The method comprises contacting the
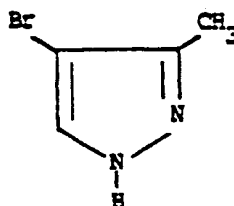cell with an amount of a molecule having the structure:

20



25

effective to decrease the expression of G-CSF by the
cell.

Further, the invention concerns a method of decreasing
the .formation of neutrophils and effecting the
metabolic functions of neutrophils, such as superoxide
or peroxide formation, which has implications for the
treatment of inflammatory and autoimmune disorders in a
human being who (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising administering
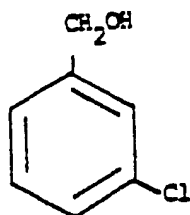to the human being an amount of a molecule having the
structure:



effective to decrease expression of G-CSF by, and thus
decrease the formation and affect the metabolic
functions of neutrophils in, the human being.

Moreover, the invention provides a method of decreasing
the expression of a mammary tumor virus, such as a
mouse mammary tumor virus, by a cell which (i)
comprises DNA encoding, and (ii) is capable of
expressing, a mammary tumor virus. The method
comprises contacting the cell with an amount of a
molecule having the structure:

78

effective to decrease the expression of a mammary tumor
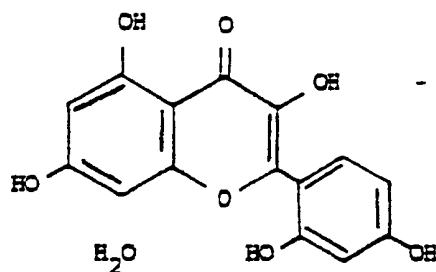virus by the cell.

Finally, the invention also provides a method of
suppressing the proliferation of a mammary tumor virus
in a subject who (i) comprises DNA encoding, and (ii)
is capable of expressing, a mammary tumor virus,
comprising administering to the subject, e.g. a human
subject or an animal subject, an amount of a molecule
having the structure:



effective to suppress the proliferation of a mammary
tumor virus in the subject.

This invention is illustrated in the Experimental Details
and Results sections which follow. These sections are
set forth to aid in an understanding of the invention but
are not intended to, and should not be construed to,

limit in any way the invention as set forth in the claims which follow.

EXPERIMENTAL DETAILS


MATERIALS AND METHODS


5      A. Cell Culture


All media and reagents used for routine cell culture were
purchased from Gibco (Grand Island, NY), Hazelton
(Lenexa, KS), or Whittaker M.A. Biologicals
10     (Walkersville, MD). Fetal calf serum (FCS) was from
Hyclone (Logan, UT), and nutrients used for serum-free
defined media were purchased from Sigma (St. Louis, MO),
Boehringer Mannheim (Indianapolis, IN), Bachem (Torrance,
CA) and Collaborative Research (Bedford, MA).
15

NIH/3T3 fibroblast cells (ATCC number CRL 1658) were used
for transfection of plasmids containing the mouse mammary
tumor virus (MMTV) promoter linked to firefly luciferase
coding sequences (see below). Cells were propagated in
20     Dulbecco's modified Eagle's medium (DMEM) obtained from
Gibco, Grand Island, NY and supplemented with 10% FCS.
For high-throughput (HTP) screening, transfected NIH/3T3
clones were transferred to serum free defined medium
consisting of Iscove's modified Eagle's medium (IMEM) and
25     Ham's F12 medium (1:1) supplemented with growth factors,
hormones and nutrients as described previously (43).


A rat pituitary cell line, designated GC, (4, 25) was
used for transfection of plasmids containing the human
30     growth hormone promoter (see below) and was maintained in
DMEM and Ham's F12 medium (1:1), supplemented with 12.5%
FCS. For HTP screening, transfected GC clones were
transferred to serum free defined medium consisting of
DMEM and Ham's F12 medium (1:1) supplemented with growth
35     factors, hormones and nutrients as described previously

(17, 5).

A human bladder carcinoma cell line (U5637, ATCC number
HTB 9) was used for transfection of plasmids containing
5     the human Granulocyte-Colony Stimulating Factor (G-CSF)
promoter (see below) and was maintained in RPMI medium
supplemented with 10% FCS.     For HTP screening,
transfected 5637 clones were transferred to a serum free
defined medium identical to that used for the NIH/3T3
10    clones.

G418 (Geneticin, Gibco) at 0.2 mg/ml was routinely added
to  both  serum  and  serum  free  defined  media  for
maintenance of cell lines transfected with the neomycin
15    resistance gene.

·B.    Plasmid  Construction  and  Molecular  Cloning  of
       Promoter-Reporter   Fusion   Constructs   to   be
       Transfected into Cells Used for a 2,000-Chemical
20     Transcription Screen

This section describes (a) the molecular cloning of the
human G-CSF promoter and adjacent 5' transcriptionally
modulatable regulatory sequences and (b) the making of
25    constructs where these regulatory sequences or those of
the human growth hormone (hGH) gene or those of the mouse
mammary tumor virus (MMTV) long terminal repeat (LTR)
control the expression of the firefly luciferase gene.
These constructs were transfected into cells as described
30    in Section C and used for a high-throughput pilot screen
of  2,000 chemicals to identify chemicals acting as
specific transcriptional modulators (see Section E and
"Results").

35    Unless   otherwise   indicated   cloning   procedures   were

performed essentially according to Maniatis et al. (1982)
(28). Oligonucleotides were synthesized by the
beta-cyanoethyl phosphoramidite method according to
protocols provided by the manufacturer of the
5    DNA-synthesizer (Model 380A, Applied Biosystems (Foster
City, CA).


1. Construction of the MMTV Promoter-Luciferase fusion
plasmid (pMluci)

10

The firefly luciferase gene was removed from the plant
expression plasmid pDO432 (33) (Figure 1) as a 1.9 kb
BamHI fragment and cloned into the BamHI site of pSVL
(Pharmacia, Piscataway, NJ), a mammalian expression
15   vector containing the SV40 promoter. The resulting
plasmid (pSVLuci; Figure 2) was digested with XhoI and
SalI to produce a 2.4 kb fragment containing the
luciferase coding sequences and the SV40 late
polyadenylation site. This fragment was inserted into
20   the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a
eucaryotic expression vector containing the MMTV
promoter. The resulting MMTV promoter-luciferase fusion
plasmid (pMLuci; Figure 3) was used to transfect NIH/3T3
cells as described below (section C1). Similar
25   constructs can be made using luciferase vectors from
Clontech (Palo Alto, CA).


2. Construction of the human growth hormone (hGH)
promoter-luciferase fusion plasmid

30

The SalI-XhoI fragment of pSVLuci (Figure 2) containing
the luciferase coding sequences and the SV40 late
polyadenylation site was inserted into pUC 8 (Biorad,
Richmond, CA), which had been linearized by a SmaI/HincII
35   digestion and ligated to XhoI linkers (New England

Biolabs, Beverly, MA). The new plasmid thus generated
(pUXLuci; Figure 4) was linearized by XhoI digestion
followed by incubation with the Klenow fragment of E.
coli DNA polymerase and the four deoxyribonucleotides to
5      fill in the single-stranded ends of the vector. This
linear (5.1 KB) form of pUXLuci was then ligated to the
filled-in 550bP HindIII-XbaI fragment of the plasmid
phGH:CAT (Figure 5) (25). Human growth hormone promoter
sequences located on the HindIII-XbaI fragment were thus
10     fused to the luciferase coding sequences located on
pUXLuci generating the plasmid phGH-Luci (Figure 6),
which was used in transfections of GC cells as described
below (Section C2).

15     3.   Construction   of   the   human   Granulocyte-Colony
stimulating factor (hG-CSF) Promoter-Luciferase fusion
plasmid (pG-Luc1)

Information on the G-CSF upstream and coding sequences
20     was published by Nagata et al. (1986) and was used to
synthesize 5 oligonucleotide probes (OL-1 to OL-5) to
screen a human leukocyte genomic DNA library (Clontech,
Palo Alto, CA) according to the supplier's instructions.
The sequences of the oligonucleotide probes were:

25

          5' GCTTTTTGTTCCAACCCCCCTGCATT 3' (OL-1);
          5' CCCTGCATTGTCTTGGACACCAAAT 3' (OL-2);
          5' GCGCTCCAGGAGAAGCTGGTGAGT 3' (OL-3);
          5' AAGCTGATGGGTGAGTGTCTTGGC 3' (OL-4);
30        5' ATCAGCGGCTCAGCCTTCTT 3' (OL-5);

The sequences of OL-1, OL-2 and OL-5 recognize the G-CSF
promoter region, OL-4 recognizes the first intron/exon
junction and OL-3 recognizes sequences within the second
35     exon (32). One of the clones isolated from the leukocyte

library using these oligonucleotide probes contains a 3.5 kb SalI-BamHI fragment of G-CSF genomic sequence consisting of 3.3 kb of promoter sequence and two hundred base pairs of the coding region. This fragment was inserted into the vector pGEM-7-Zf (Promega, Madison, WI) which had previously been digested with SalI/ BamHI, resulting in the vector pJM710 (Figure 7). pJM710 was then digested with PstI, and the resulting 1.6 kb fragment containing G-CSF upstream sequences and the first 15 bases of the G-CSF leader sequence was inserted into the PstI site of pGEM5-Luci (Figure 8) to generate pG-Luc1 (Figure 9). This construct was then used for transfections of 5637 human bladder carcinoma cells as described below in section C3. pGEM5-Luci (Figure 8) had previously been constructed by inserting the XbaI/SalI fragment from pSVLuci (Figure 2) containing the luciferase coding sequence and the SV40 late polyadenylation signal into pGEM 5-Zf (Promega, Madison WI) digested with XhoI/SalI.

## C. Construction of Single Cell Clones Containing Various Promoter-Luciferase Fusion Constructs

### 1. pMluci

pMluci (Figure 3) and pSV2Neo, an antibiotic resistance plasmid (34), were co-transfected into NIH/3T3 mouse fibroblast cells using the calcium phosphate precipitation method (15) with a commercially available kit (Pharmacia, Piscataway NJ). Two days later, cells were transferred to media containing 0.4 mg/ml G418 and were grown for an additional 10-14 days. G418-resistant clones were isolated by standard methods. Once sufficient cell numbers were obtained, clones were analyzed based on several criteria: constitutive

luciferase production, induction of luciferase expression
by dexamethasone (1 µM Sigma, St. Louis, MO),
satisfactory attachment to microtiter plates used in the
high-throughput screen (see section E) and acceptable

5      standard deviation in multiple luciferase expression
assays (see below for assay protocol). This analysis was
carried out using the luciferase assay conditions
described in sections D and E provided herein. Of the
clones which satisfied the above criteria for the high

10     throughput screen, one clone, M10, was selected for use.


       2. phGH-LUCI


15     phGH-LUCI (Figure 6) and pRSVNeo, an antibiotic
resistance plasmid (14), were co-transfected into GC rat
·pituitary cells as described above. Selection of
G418-resistant cell clones was described above except for
using a concentration of 0.2 mg/ml G418. Analysis of the

20     cell clones was performed as above, except that known
inducers of hGH expression (10-100 nM rat growth hormone
releasing factor (rGRF, Bachem, Torrance, CA) and 10µM
·forskolin (Sigma, St. Louis, MO) were used in place of
dexamethasone. One clone, 532, was selected for further

25     use in the high throughput screen.


       3. pG-LUC1


pG-LUC1 (Figure 9) and pRSVNeo were co-transfected into

30     5637 human bladder carcinoma cells as described above.
Selection of G418 resistant clones was as described above
except for using a concentration of 0.1 mg/ml G418.
Analysis of cell clones was performed as above except
that a known inducer of G-CSF expression (1-5 µg/ml

35     lipopolysaccharide (LPS), E. coli serotype 055:b5, Difco,

Detroit, MI or Sigma, St. Louis, MO) was used in place of dexamethasone. One clone, G21, was selected for use.

## D. Liquid Scintillation Counter Bioluminescence Assay

To assay for luciferase expression in transient expression assays in the various transfected clones, cells were incubated with various transcriptional inducers in serum free defined media, washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS, Gibco) and lysed and measured according to the method of DeWet, et al. (100). Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer (see section E).

## E. High-Throughput (HTP) Screening

Cell plating: 96 well plates were treated with 50 μl per well of human fibronectin (hFN, 15 μg/ml in PBS, Collaborative Research, Bedford, MA) overnight at 37C. hFN-treated plates were washed with PBS to remove excess hFN prior to cell plating. M10, 532, and G21 cells maintained in their respective serum media (with 0.2 mg/ml G418) were washed with PBS, harvested by trypsinization, and counted using a hemocytometer and the Trypan Blue exclusion method according to protocols provided by Sigma, St. Louis, MO Chemical Company. Cells were then diluted into serum free defined media (with 0.2 mg/ml G418), and 0.2 ml of cell suspension per well was plated onto microtiter plates (532 and G21) or hFN-treated plates (M10). Plates were incubated overnight at 37C in a humidified 5% $CO_2$ atmosphere.

Addition of Chemicals to Cells: Chemicals from the

Oncogene Science file were dissolved in DMSO at
concentrations of 3-30 mg/ml and diluted 5 fold, 110
fold, and 726 fold. 10 $\mu$l of each dilution were added to
each of quadruplicate samples of cells contained in the
5      wells of 96-well microtiter plates. Cell plates were
then shaken and incubated for 6 hours at 37C, 5% $CO_2$.


Bioluminescence Assay: After incubation with OSI-file
chemicals, cell plates were washed 3 times with PBS.
10     Cells were lysed and bioluminescence was measured in a 96
well luminometer according to the method of DeWet, et al.
(100). Data were captured using Lotus-Measure (Lotus)
software and processed by custom-designed macros written
in Lotus.

15


·F. Isolation of Total Cellular RNA


Total cellular RNA was isolated from the G21 cell clone
20     or from untransfected 5637 cells following incubation for
6 hours with various transcriptionally modulating
chemicals identified in the high-throughput screen.
Cells were grown in serum free medium as described above.
Total cellular RNA was isolated using the RNAZol method
25     (CINNA/BIOTECX, Friendswood, TX, Laboratories
International, Inc.). Cells were resuspended and lysed
with RNAZol solution (1.5 ml/9 cm petri dish) and the RNA
was solubilized by passing the lysate a few times through
a pipette. Chloroform was added to the homogenate (0.1
30     ml/1 ml), and samples were shaken for 15 seconds followed
by a 5 minute incubation on ice. After centrifuging for
10 minutes, the upper phase was collected and an equal
volume of isopropanol was added. Samples were incubated
for 45 minutes at -20C, and the RNA was pelleted for 15
35     minutes at 12,000 x g at 4C. The RNA pellet was then

washed with 70% ethanol and dried briefly under vacuum.

G.  Northern Blotting

5       Total cellular RNA was isolated from 5637 cells following
        incubation with chemicals as described above and
        electrophoresed in a 1% Agarose-Formaldehyde gel. The
        RNA was transferred to Duralon-UV nylon filters
        (Stratagene, La Jolla, CA) using the manufacturer's
10      recommended protocol. The filters were prehybridized for
        4 hours (prehybridizing solution = 5X SSC, 50 mM sodium
        pyrophosphate, 10X Denhardt's solution, 10% dextran
        sulfate, 7% SDS and 250 1g/ml denatured ssDNA) and then
        hybridized in the same solution for 16 hours at 65C in
15      the presence of G-CSF or Beta-Actin (Oncor, Gaithersburg,
        MD) specific probes. The G-CSF probe was a 0.6kb AflII
        to XhoI fragment which contained most of exon 5 of the
        human G-CSF gene. The B-actin probe was used as a
        control probe to normalize for the total amount of RNA.
20      The probes were labeled with alpha-$^{32}$P dCTP using a
        random primed DNA labeling kit (Amersham, Arlington, IL).
        Following hybridization, filters were first probed with,
        G-CSF-and Fusion reprobed with B-Actin Probe were washed
        three times at room temperature with 1X SSC, 0.13% SDS
25      and three times at 65C with 0.2X SSC, 0.1% SDS. Filters
        were first probed with G-CSF-and then reprobed with
        B-actin-probe. Exposure to x-ray films was performed
        overnight. Bands were excised and counted in a liquid
        scintillation counter (LKB, Gaithersburg, MD), and counts
30      obtained with the G-CSF specific probe were normalized
        relative to the counts obtained with the B-Actin specific
        probe.

35      H. S1 Nuclease protection

S1 Nuclease protection assays were carried out as described in reference 94.

5     I. MTT Cell toxicity assay

To determine cytotoxic concentrations of chemicals registering as positives in the High-Throughput luciferase assay the MTT cytotoxicity assay was employed

10     (95). In this assay, a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT] is reduced to a colored formazan product by reducing enzymes present only in living metabolically active cells.

15

J. Two-antibody Sandwich Immunoassay

Supernatants from 5637 bladder carcinoma cells incubated with chemicals registering as positives in the G-CSF

20     promoter/luciferase High-Throughput assay were assayed for secreted G-CSF protein using the two-antibody sandwich immunoassay (96). The G-CSF Assay kit manufactured by Oncogene Science, Inc. was used and the manufacturer's instructions were followed.

25

K. Construction of a Yeast Expression Vector

Plasmid pHZ18 (97,98) contains 2μ DNA for propagation in S. cerevisiae, the yeast promoter cyc 1, which is

30     compatible with expression in yeast cells, and the URA 3 gene for selection. The plasmid was linearized with BamH1, the ends were filled-in using deoxynucleotides and E.coli DNA polymerase Klenow fragment, and then the plasmid was digested with Aat II. A 4.1 kb fragment

35     containing the cyc1 promoter, URA3 and 2μ genes was

separated by agarose gel electrophoresis and subsequently purified by electroelution onto ion-exchange paper (Whatman, DE81). Plasmid pBR322 was treated with endonucleases Aat II and Pvu II and a 2.2 kb fragment

5    containing the plasmid's origin of replication and the amp$^R$ gene was isolated by agarose gel electrophoresis and eluted onto DE81 paper.

The 2.2 kb pBR322 and 4.1 kb pHZ18 fragments were ligated

10   using T4 DNA ligase according to standard procedures (94). The resulting 6.3 kb vector pHZBR was digested with BamH1 for subsequent insertion of the luciferase coding sequence downstream of the cyc1 promoter.

15   An Nco I - Sal I restriction fragment containing the luciferase gene starting at the second ATG, was made blunt-ended by filling in and ligated into the filled-in BamH1 site of pHZBR as readily understood by one skilled in the art. Clones of the correct orientation were

20   identified via restriction mapping to yield plasmid pHZluci24. This plasmid was used to transform S.cerevisiae strain DB745.

L. Transformation of Yeast Cells

25

S.cerevisiae DB745 were made competent according to published methods (99). One and 4µg of either pHZluci24 or pHZ18 (transfection control) were added to the competent cells and incubated at 30°C for 30 minutes.

30   Lithium acetate-PEG was mixed gently with the cells and allowed to incubate for another 45 minutes at which time the cells were shifted to 42°C for 5 minutes. The cells were spread onto uracil(-) plates and incubated at 30°C for several days. Cell colonies were picked, grown to

35   saturation in YPD media and analyzed for luciferase

activity (99) Cell lysis and analysis of luciferase
activity can be carried out by methods known to one
skilled in the art (99, 100). Stock cultures were made
from clones expressing luciferase positive clones.

5      RESULTS


A. Validation of Cell Lines


10     Prior to initiation of drug screening, it was
demonstrated that the transfected promoter-luciferase
fusion plasmids were reacting to transcriptional inducers
in a manner as predicted based on the published
literature. As shown in Figure 10, all three transfected
15     cell clones chosen responded to inducers which have been
reported to stimulate the endogenous genes; the
·MMTV-luciferase containing clone M10 was stimulated 11.6
fold by 1μM dexamethasone, the hGH-luciferase containing
clone 532, was stimulated 2.2 fold by 100 nM rat growth
20     hormone releasing factor (GRF), and the hG-CSF containing
clone G21 was stimulated 5.7 fold by 5 μg/ml
lipopolysaccharide (LPS).


25     It was also demonstrated that certain steroidal chemicals
other than dexamethasone modulated luciferase expression
in the cell clone M10, which harbors the MMTV
promoter-luciferase fusion construct. As shown in Figure
11, dexamethasone stimulated the MMTV promoter in cell
30     clone M10 (mouse fibroblast origin), while progesterone
did not. It has been shown that a rat fibroblast cell
line which contains high levels of glucocorticoid
receptor but low levels of progesterone receptor, shows
stimulation of the MMTV promoter by the glucocorticoid
35     dexamethasone but not by progesterone (7). In addition,

92

Figure 11 shows that the mineralocorticoid aldosterone
stimulates clone M10, as is expected based on previously
published work which indicates that aldosterone can act
through the glucocorticoid receptor to stimulate the MMTV
5       promoter (6).

A 7-hour incubation of G 21 cells with PMA in serum-free
media increased luciferase expression directed by the G-
CSF promoter by 34.6 fold in the absence and by 24.6 fold
10      in the presence of EGF. TNF-Alpha induction did not
significantly change on EGF addition (2.8 fold with and
2.1 fold without EGF).

B. High-Throughput Drug Screen
15

Table 1 shows a summary of the results of a one-week,
high-throughput screen of 2,000 chemicals to identify
those chemicals specifically stimulating or inhibiting
transcription from the G-CSF, hGH or MMTV promoters.
20      This screen concurrently tested chemicals at three
concentrations on quadruplicate samples of the M10, 532
and G21 cell lines. A minimum stimulation of one
promoter, to the degree indicated, and less than 50%
activation of the other two promoters was required for

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## TABLE 1

5

SUMMARY OF HIGH-THROUGHPUT SCREEN

10

Number (%) of Chemicals Which Activate Expression:

| Promoter | 2-3 Fold | 3-5 Fold | 5-7 Fold | 7-10 Fold | >10 Fold | Total |
|---|---|---|---|---|---|---|
| G-CSF | NA | 23 (1.1%) | 10 (0.5%) | 3 (0.15%) | 2 (0.10%) | 38 (1.9%) |
| hGH | NA | NA | 12 (0.6%) | 5 (0.03%) | 6 (0.03%) | 23 (1.14%) |
| MMTV | 15 (0.7%) | 1 (0.05%) | 0 (0%) | 1 (0.05%) | 1 (0.05%) | 18 (0.9%) |

25

Number (%) of Chemicals Which Inhibit Expression >3 Fold

Promoter

30

| G-CSF | 7 | (0.35%) |
|---|---|---|
| hGH | 42 | (2.1 %) |
| MMTV | 1 | (0.05%) |

35

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## TABLE 2

A) TRANSCRIPTIONAL ACTIVATORS

| Chemical # | Chemical Name | FOLD INDUCTION RELATIVE TO SOLVENT CONTROL | | |
|---|---|---|---|---|
| | | GCSF | hGH | MMTV |
| G-CSF: | | | | |
| 40 | 3-Acetyl-2-6-Bis(tertiary butyl amino)-4-methyl-pyridine | 5.62 | 0.62 | 0.27 |
| 58 | 1-Acetylimidazole | 6.03 | 0.17 | 0.42 |
| 237 | N-Carbethoxy-phthalimide | 4.77 | 0.06 | 0.62 |
| 254 | 1-(2-Chloroethyl)piperidine | 4.09 | 0.90 | 0.98 |
| 364 | Melamine | 3.67 | 1.18 | 1.07 |
| 473 | 1,3,5,-Triazine | >3 | 0.50 | 0.87 |
| 542 | 5-Bromo-2'-deoxycytidine | 6.28 | 1.08 | 1.26 |
| 543 | 5-Bromo-2'-deoxyuridine | 7.17 | 0.72 | 0.98 |
| 878 | Blueberry leaf extract | 3.84 | 1.17 | 0.78 |
| 1025 | Culvers Root extract | 4.09 | 0.98 | 1.24 |
| 1234 | 4-Aminocinnamic Acid hydrochloride | 4.97 | 0.51 | 1.03 |
| 1255 | 1-Bromo-3,5-dichlorobenzene | 6.74 | 0.43 | 1.09 |
| 1374 | 4'-Amino-N-methylacetanilide | 11.03 | 0.05 | 1.05 |
| 1375 | 4'-(aminomethyl)benzene sulfonamide hydrochloride | 8.94 | 0.04 | 1.37 |
| 1376 | 2-Amino-5-Methyl benzene sulfonic acid | 6.37 | 0.04 | 1.32 |
| 1397 | 5-Amino-3-methylisothiazole hydrochloride | 3.63 | 0.57 | 1.13 |
| 1482 | 2-Aminophenyl disulfide | 3.98 | 0.54 | 1.07 |
| 1483 | 4-Aminophenyl disulfide | 4.64 | 0.38 | 1.09 |

TABLE 2 (CONT.)

| | Chemical# | Chemical Name | FOLD INDUCTION | | |
|---|---|---|---|---|---|
| | | | GCSF | hGH | MMTV |
| 5 | 1521 | 2-Amino-6-purinethiol | 3.59 | 0.73 | 0.92 |
| 10 | 1583 | 8-Bromoadenosine | 5.82 | 0.12 | 0.88 |
| | 1592 | Bis(2,2,3,3,4,4,5,5,6,6,7,7,) dodecafluoroheptyl-(+)-camphorate | 3.20 | 0.74 | 1.34 |
| 15 | 1783 | Cupferron | 6.55 | 0.32 | 0.89 |
| | 1793 | Cyanomethyl-N,N-dimethyl dithiocarbamate | 8.50 | 0.52 | 1.21 |
| 20 | 1994 | 3-Bromobiphenyl | 3.29 | 0.34 | 0.83 |
| | 2001 | 1-Bromo-4-tertiary butyl benzene | 3.11 | 0.74 | 1.12 |
| | 2030 | 4-Bromo-2-fluoro-6-nitroanizol | 5.53 | 0.67 | 0.87 |
| 25 | 2096 | (+)-1-Bromo-3-Chloro-2methyl propane | 3.27 | 0.61 | 0.89 |
| | 2097 | 1-Bromo-5-Chloro pentane | 5.09 | 0.88 | 1.22 |
| 30 | 2129 | 4-Chlorobenzyl Chloride | 3.23 | 0.75 | 0.95 |
| | GROUP A: | | | | |
| 35 | 378 | 7-Oxo-7H-benzo[e]perimidine 4-carboxylic acid | 4.12 | 0.26 | 0.59 |
| | 423 | Quinacrine dihydrochloride hydrate | 2.39 | 0.56 | 0.84 |
| 40 | 427 | Resazurin | 3.14 | 0.43 | 0.71 |
| | 838 | Thionin | 3.20 | 0.23 | 0.58 |
| | 1776 | Cresyl Violet Acetate | 3.50 | 0.15 | 1.36 |
| 45 | 1904 | 9-Aminoacridine hydrochloride | 4.12 | 0.54 | 0.82 |
| | GROUP B: | | | | |
| | 670 | Methyl Green | >3 | 0.52 | 0.79 |
| 50 | 1780 | Crystal Violet | 20.39 | 0.38 | 1.15 |

TABLE 2 (CONT.)

| Chemical# | Chemical Name | FOLD INDUCTION | | |
|---|---|---|---|---|
| | | GCSF | hGH | MMTV |
| **GROUP A AND B:** | | | | |
| 80 hGH: | Acridine Orange | 5.57 | 0.65 | 0.63 |
| 70 | 2-Acetylpyrrole | 0.43 | 8.25 | 0.85 |
| 299 | 10,11-Dihydrocarbamazepine | 0.53 | 5.46 | 0.47 |
| 322 | 1-ethyl-2-benzimidazolinone | 0.60 | 11.18 | 1.12 |
| 325 | Fisetin | 0.14 | 5.42 | 1.0 |
| 552 | 3-(4-chlorophenyl)-1-methoxy-1-methyl urea | 0.81 | 5.31 | 0.86 |
| 790 | Rivanol | 0.01 | 5.94 | 0.58 |
| 792 | Rose Bengal | 0.94 | 5.31 | 1.21 |
| 855 | Tripalmitin | 0.28 | 6.49 | 0.42 |
| 1004 | Arnica 4x | 0.85 | 6.48 | 1.22 |
| 1150 | Rochester # 6150 | 0.38 | 5.79 | 0.80 |
| 1251 | Bromocresol Green | 0.14 | 15.19 | 0.33 |
| 1337 | 4-Amino-5-hydroxy-1-naphthalene sulfonic acid | 0.07 | 15.87 | 0.23 |
| 1499 | 2-Amino-4-phenylthiazole hydrobromide monohydrate | 0.24 | 5.55 | 0.61 |
| 1550 | 2-Aminothiazole | 0.04 | 5.44 | 0.87 |
| 1552 | 2-amino-2-thiazoline | 1.23 | 7.26 | 0.52 |
| 1561 | 4-Amino-3,5,6-trichloropicolinic acid | 0.23 | 8.05 | 0.48 |
| 1598 | N,N'-Bis-[3-(4,5-dihydro-1H-imidizol-2-yl)phenyl] urea dipropanoate | 0.72 | 5.32 | 1.27 |

<u>TABLE 2 (CONT.)</u>

|  | Chemical# | Chemical Name | FOLD INDUCTION | | |
| --- | --- | --- | --- | --- | --- |
|  |  |  | GCSF | hGH | MMTV |
| 5 | 1578 | 4,8-Bis(hydroxymethyl)-tricyclo [5,2,1,0^{2.6}]decane | 0.36 | 7.08 | 0.89 |
| 10 | 1740 | 5-carbethoxy-2-thiouracil | 0.74 | 17.77 | 0.87 |
|  | 1747 | N_6-carbobenzyloxy-L-lysine | 0.78 | 6.16 | 0.86 |
| 15 | 1804 | Cyclobutane carboxylic acid | 1.05 | 9.41 | 0.49 |
|  | 1876 | Alec Blue | 0.87 | 11.91 | 0.40 |
|  | 1881 | Alizarin Blue Black B | 0.21 | 18.87 | 0.69 |
| 20 | MMTV: |  |  |  |  |
|  | 189 | Bathocuproinedisulfonic Acid disodium salt hydrate | 1.06 | 1.47 | 2.80 |
| 25 | 453 | 2,2':6',2"-Terpyridine | 0.79 | 0.58 | 13.30 |
|  | 519 | b-Apo-8'-carotenal | 1.15 | 0.68 | 2.76 |
| 30 | 582 | Copaiva Balsam | 1.10 | 0.15 | 2.34 |
|  | 629 | Homoveratric acid | 0.85 | 1.05 | 2.48 |
| 35 | 633 | 5-Iodorotic acid | 1.02 | 0.86 | 2.46 |
|  | 765 | Prednisolone-21-Acetate | 0.96 | 1.30 | 2.68 |
|  | 828 | 2,4,5,4'-Tetrachlorodiphenylsulfide | 1.47 | 1.34 | 2.20 |
| 40 | 848 | Triamcinolone acetonide | 0.75 | 1.28 | 2.43 |
|  | 944 | Peanut | 1.15 | 0.91 | 2.10 |
| 45 | 1259 | 5-Amino-4,6-dichloropyrimidine | 0.72 | 0.91 | 2.18 |
|  | 1316 | 2-Aminofluorene | 0.74 | 1.39 | 2.33 |
|  | 1318 | 2-Amino-9-fluorenone | 1.13 | 0.85 | 2.41 |
| 50 | 1384 | 2-Amino-4'-methylbenzophenone | 1.33 | 0.50 | 2.43 |

## TABLE 2 (CONT.)

|  |  |  | FOLD INDUCTION | | |
|---|---|---|---|---|---|
| | Chemical# | Chemical Name | GCSF | hGH | MMTV |
| | 1573 | 5-Bromoacenapthene | 1.49 | 0.34 | 4.30 |
| | 2064 | 4-(Bromomethyl)-6,7-dimethoxy-coumarin | 0.82 | 1.10 | 2.53 |
| | 2148 | 2-chlorocyclohexanone | 0.45 | 0.92 | 2.82 |
| | 2191 | Chloramphenicol | 0.37 | 0.35 | 7.32 |

B) TRANSCRIPTIONAL INHIBITORS

|  |  |  | FOLD INHIBITION RELATIVE TO SOLVENT CONTROL | | |
|---|---|---|---|---|---|
| | Chemical# | Chemical Name | GCSF | hGH | MMTV |
| | G-CSF: | | | | |
| | 209 | 4-Benzoylpyridine | 6.66 | 1.08 | 0.81 |
| | 371 | Morin hydrate | 11.11 | 0.41 | 0.89 |
| | 650 | Maclurin | 10.0 | 0.34 | 1.04 |
| | 798 | Salicylamide | 4.76 | 0.90 | 0.88 |
| | 2009 | 4-Bromo-3,5-dimethylpyrazole | 3.70 | 0.57 | 0.64 |
| | 2082 | 4-Bromo-3-Methylpyrazole | 5.26 | 0.65 | 1.23 |
| | 2121 | 3-Chlorobenzyl alcohol | 4.76 | 0.40 | 1.14 |
| | hGH: | | | | |
| | 183 | Auramine O | 0.72 | 4.00 | 0.70 |
| | 240 | Carminic acid | 0.63 | 5.26 | 0.80 |
| | 443 | Sulfamethazine | 0.60 | 4.76 | 0.79 |
| | 512 | Amaranth | 0.81 | 5.26 | 0.88 |

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## TABLE 2 (CONT.)

| | Chemical# | Chemical Name | FOLD INHIBITION | | |
|---|---|---|---|---|---|
| | | | GCSF | hGH | NPTY |
| | 541 | 5-Bromo-4-Chloro-3-indoxyl-phosphate K-salt | 0.90 | 6.25 | 0.86 |
| | 556 | Chromazurol S | 0.73 | 33.33 | 0.87 |
| | 561 | Clove Oil | 0.62 | 5.00 | 0.05 |
| | 577 | Na-Ne-Diacetyl-L-lysine | 0.64 | 4.00 | 0.88 |
| | 578 | Dibenzoyl-D-tartaric acid | 0.85 | 4.00 | 0.91 |
| | 630 | Hydantoin-5-acetic acid | 0.70 | 3.57 | 0.74 |
| | 840 | Kernechtrot | 0.84 | 5.00 | 0.59 |
| | 759 | Piperidine | 0.64 | 5.88 | 0.95 |
| | 764 | Prednisolone | 0.82 | 4.54 | 0.59 |
| | 875 | Black Walnut extract | 0.69 | 6.25 | 0.80 |
| | 892 | Colts Foot Leaves extract | 0.88 | 11.11 | 0.87 |
| | 893 | Comfrey Leaf extract | 0.74 | 11.11 | 0.90 |
| | 920 | Horehound Herb extract | 0.56 | 3.84 | 0.84 |
| | 921 | Horsetail Grass extract | 0.72 | 3.44 | 0.86 |
| | 942 | Pau D'Arco extract | 0.80 | 6.25 | 0.63 |
| | 970 | Thyme extract | 0.57 | 4.34 | 1.07 |
| | 1591 | 1,2-Bis(di-p-tolylphosphino)-ethane | 0.56 | 5.55 | 0.96 |
| | 1604 | 2,4-Bis[5,6-bis(4-sulfophenyl)-1,2,4-Triazine-3-yl]-pyridine, tetrasodium salt hydrate | 0.77 | 5.00 | 0.97 |
| | 1635 | [(1S)-endo]-(-)-Borneol | 0.71 | 9.09 | 0.99 |
| | 1840 | 1,2-Bis(2-pyridyl)-ethylene | 0.79 | 5.00 | 0.59 |
| | 1841 | 2,3-Bis(2-pyridyl)-pyrazine | 0.83 | 5.55 | 0.60 |

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## TABLE 2 (CONT.)

| | | FOLD INHIBITION | | |
|---|---|---|---|---|
| Chemical# | Chemical Name | GCSF | hGH | MMTV |
| 1648 | 2-[5,6-Bis(4-sulfophenyl)-1,2,4-triazine-3-yl)-4-(4-sulfophenyl)-pyridine, trisodium salt | 0.86 | 7.69 | 1.00 |
| 1651 | Bis(2,2,2-trifluoroethyl) (methocarbonyl-methyl)-phosphonate | 0.59 | 3.57 | 0.70 |
| 1655 | 2,5-Bis(trifluoro-methyl)benzoic acid | 0.54 | 4.76 | 0.81 |
| 1703 | 3-Bromobenzonitrile | 0.76 | 10.00 | 0.90 |
| 1704 | 4-Bromobenzonitrile | 0.77 | 4.16 | 0.94 |
| 1705 | 4-Bromobenzophenone | 0.54 | 14.28 | 0.62 |
| 1712 | Calcein Blue | 0.74 | 8.33 | 0.94 |
| 1720 | (1S)-(-)-Camphor | 0.55 | 4.76 | 0.56 |
| 1764 | 7-(Carboxymethoxy)-4-Methylcoumarin | 0.55 | 7.14 | 0.82 |
| 1770 | Carminic acid | 0.54 | 10.00 | 0.57 |
| 1771 | L-Carnosine | 0.71 | 10.00 | 0.72 |
| 1773 | O-Cresolphthalein Complexone | 0.62 | 10.00 | 0.67 |
| 1890 | Alloxazine | 0.80 | 5.25 | 0.58 |
| 2035 | 5-Bromofuroic acid | 0.57 | 7.14 | 0.89 |
| 2036 | 8-Bromoguanosine | 0.58 | 4.34 | 0.81 |
| 2037 | 1-Bromohexadecane | 0.51 | 4.00 | 0.50 |
| MMTV: | | | | |
| 2010 | 2-Bromo-4,6-dinitroaniline | 0.80 | 0.63 | 3.57 |

a chemical to be considered a selective activator. A
minimum inhibition of 3 fold of one promoter and less
than 20% inhibition of the other two promoters was
required for a chemical to be considered a selective
inhibitor. Table 2 gives the names and induction or
inhibition ratios of the lead chemicals identified for
each promoter. Figure 12 illustrates the transcriptional
stimulation and Figure 13 the transcriptional inhibition
observed with some of the lead chemicals. Some of the
chemicals activating G-CSF transcription fell into
conspicuous groups of analogs (Table 2; Group A and B).
Although not specifically indicated in Table 2, groups of
homologs and analogs can also be found for
G-CSF-inhibiting as well as hGH-activating chemicals.

To determine the number of lead chemicals, which
reproducibly score as positives in repeated luciferase
assays, two types of experiments were conducted:

1) G-CSF lead chemicals #1780, #58, #1783, #1374 were
subjected to 48 independent luciferase assays performed
on the same day. Compounds #58, #1780 and #1374 scored as
positives in every single one of these assays inducing
luciferase expression between 2 and 28-fold (#58), 20 and
80-fold (#1780) and 5 and 40-fold (#1374). Compound
#1783 scored as positive only in half of the 48 repeat
assays probably due to its relatively low induction of
luciferase expression (1.5 to 8-fold).

2) All of the 18 lead chemicals inducing luciferase
expression from the MMTV promoter were again subjected to
luciferase assays: 10 chemicals (#453, #519, #562, #765,
#828, #848, #1269, #1316, #1384 and #2148) again induced
luciferase expression between 2.1 and 2.8-fold. Probably
due to the relatively low induction level close to the

background of the assay, the other eight lead chemicals
did not repeat on that particular day. The most prominent
lead chemical, #453 (13.3-fold induction in the original
high-throughput assay), was repeated in a total of 3
5    independent assays and consistently induced luciferase
expression from the MMTV promoter between 10 and 35-fold.
Replacing DMSO by methanol to dissolve the chemical did
not affect its ability to activate the MMTV promoter.

10   Aqueous clarified supernatants derived from individual
Actinomyces colonies prepared by standard methods known
to those skilled in the art as well as corresponding
methanol extracts were assayed for modulation of
luciferase expression from three target promoters of
15   interest.

Out of 356 samples tested for modulation of the three
different promoters, 25 samples scored as positives, 7 of
which were promoter-specific. A summary of the obtained
20   results is contained in Figures 52 and 53. Thus high-
throughput screening of fermentation broth samples using
a luciferase expression assay consistently leads to the
discovery of lead samples with the potential to be
developed into novel pharmaceuticals.

25

C. Effects of Lead Chemicals on Endogenous G-CSF mRNA
Levels

Northern blot analysis was used to demonstrate the
30   stimulatory effects of lead chemicals #670 and #1255 on
endogenous G-CSF mRNA levels. As shown in Figure 14,
both OSI #670 and #1255 stimulated production of G-CSF
mRNA, as shown by a G-CSF-specific probe, but not of
actin mRNA, as shown by a b-actin-specific probe. Also
35   shown are the effects of the solvent, DMSO, used to

dissolve the chemicals and a proteinaceous positive regulator, interferon-gamma. From these data it is concluded that chemicals, which induce luciferase expression from specific promoters, in plasmids stably
5      integrated into cells, are also capable of stimulating mRNA production from the corresponding endogenous promoters without using a reporter system.


        To further confirm, that compounds that had been
10     identified in a luciferase expression assay using a G-CSF specific reporter cell line would be active in inducing transcription of the endogenous G-CSF gene, cells from the parental cell line 5637 used to construct the reporter cell line were incubated with cycloheximide
15     (25 μg/ml), DMSO (0.5%,solvent control) and low, medium and high concentrations of the compounds 542 (10, 50, 250 μM),1255 (20, 100, 500 μM), 1793(0.25, 1.2, 6.25 μM) and 1904 (20, 100 μM)   for 18 hours. RNA was extracted and the concentration of G-CSF, GM-CSF and gamma-actin mRNA
20     was determined by the S1 protection method as described in Materials and Methods. The positions of G-CSF GM-CSF and gamma-actin specific protected fragments are indicated (G, GM, A) at the left side of the gel (Figure 15).

25


        Interestingly, all four compounds tested increased the amount of G-CSF mRNA.  It is of great interest that at least two of them, namely #542 and #1793, also increased the amount of GM-CSF mRNA. Compound #543, a structural
30     analog of #542 showed similar activity.


        D. Dose response Analysis of Structurally Related Lead Chemicals


35     Among the chemicals which specifically activated the

G-CSF promoter were groups of structural homologs. Three such homologs, #80, #670, and #1780, belong to groups listed in Table 2. These three structurally related chemicals all specifically activated the G-CSF promoter.

5 Dose response graphs obtained with chemicals #80, #670, and #1780 are shown in Figure 16. Although these chemicals all demonstrate large maximal stimulations, it is clear that their potencies, as measured by their $ED_{50}$'s (concentration of chemical resulting in 50%

10 maximal stimulation), show wide variability (5-70 $\mu M$)).

### E. Effects of Lead Chemicals on Target Protein Secretion

Two of the most promising lead chemicals (# 542 and

15 #1780), which were shown to stimulate levels of endogenous G-CSF mRNA as well as luciferase expression from the G-CSF promoter/luciferase fusion constructs, were further investigated for their ability to increase G-CSF secretion into the media of 5637 bladder carcinoma

20 cells incubated with the chemicals for 48 hours. The levels of G-CSF in the cell supernatants were determined by a sandwich-antibody assay as described in Materials and Methods (Figure 17).

### F. Cytotoxicity of Lead Chemical #542

25

To address the question whether the induction of G-CSF, and GM-CSF transcription by the compound #542 was a specific effect or rather a phenomenon linked to a

30 potential sensitivity of these promoters to stress exerted by toxic compounds, the concentration dependency of induction of luciferase activity in the reporter cell line G21 was compared to the concentration dependency of inhibition of respiration in FRE cells.

35

Cells were seeded into 96 well microtiterplates (20,000 cells/well) and cultured overnight. Compound #542 was added at various concentrations and the cells were incubated for 6 hours. The MTT-colorimetric assay was carried out on identically treated samples of FRE cells. Induction of luciferase reporter signal (plain line) and on inhibition of respiration (dashed line) are plotted versus the concentrations of compound (Figure 18). The ED50 for induction of luciferase activity differed from the ED50 for inhibition of respiration by a factor of almost 10, which might indicate that compound #542 exerts a specific effect on G- and GM-CSF transcription.

## G. Luciferase expression assay in yeast

To determine, whether the luciferase expression assay would also be useful to study other than mammalian cells, a yeast expression plasmid carrying the luciferase gene under control of a yeast promoter was constructed and transfected into appropriate yeast cells. Luciferase activity was demonstrated using the procedures described in Materials and Methods and stock cultures were made from position clones.

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## REFERENCES

1.  Angel, P., Baumann, I., Stein, B., Delius, H.,
    Rahmsdorf, H.J. and Herrlich, P. (1987)
    12-O-tetradecanoyl-phorbol-13-acetate (TPA)
    induction of the human collagenase gene is mediated
    by an inducible enhancer element located in the
    5'-flanking region. Mol. Cell. Biol., 7:2256.

2.  Angel, P., Imagawa, M., Chiu, R. Stein, B., Imbra,
    R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P.
    and Karin,M. (1987) Phorbol ester-inducible genes
    contain a common cis element recognized by a
    TPA-modulated trans-acting factor, Cell, 49:729.

3.  Arnheim, N. (1979) Characterization of mouse
    ribsomal gene fragments purified by molecular
    cloning, Gene, 7:83.

4.  Bancroft, F.C., Wu, G.J., and Zubay, G. (1973)
    Proc. Natl. Acad. Sci. USA, 73:29.

5.  Bottenstein, J., Hayashi, I., Hutchings, S., Masui,
    H., Mather, J., McClure, D.B., Ohasa, S., Rizzino,
    A., Sato G., Serrero, G., Wolfe, R., and Wu, R.
    (1979) The growth of cells in serum-free
    hormone-supplemented media, Methods in Enzymology,
    58:94.

6.  Cato, A.C.B. and Weinmann, J. (1988)
    Mineralcorticoid regulation of transcription of
    transfected mouse mammary tumor virus DNA in
    cultured kidney cells, J. Cell Biol., 106(6):2119.

7.   Cato, A.C.B., Miksicek, R., Schutz, G., Arnemann,
     J., and Beato, M. (1986) The hormone regulatory
     element of mouse mamary tumor virus mediates
     progesterone induction, EMBO J., 5(9):2237.

8.   De Wet, J.R., Wood, K.V., Helinski, D.R., and
     DeLuca, M. (1985) Cloning of firefly luciferase
     cDNA and the expression of active luciferase in
     Escherichia coli, Proc. Natl. Acad. Sci. USA,
     82:7870.

9.   Denison, M.S., Fisher, Springfield, NJ, J.M., and
     Whitlock, Jr., J.P. (1988) Inducible,
     receptor-dependent protein-DNA interactions at a
     dioxin-responsive transcriptional enhancer, Proc.
     Natl. Acad. Sci. USA, 85:2528.

10.  Edelman, A.M., Blumenthal, D.R. and Krebs, E.G.
     (1987) Protein Serine/Threonine Kinases Ann. Rev.
     56:567-613.

11.  Engebrecht, J.M., Simon, M., and Silverman, M.
     (1985) Measuring gene expression with light.
     Science, 227:1345.

12.  Evans, R.M. and Hollenberg, S.M. (1988) Zinc
     fingers: Gilt by assocation, Cell, 52:1.

13.  Evans, R.M. (1988) The steroid and thyroid hormone
     receptor superfamily, Science, 240:889.

14.  Gorman, C. (1985) Vectors used in mammalian cell
     expression. In DNA Cloning, Vol. II (D.M. Glover,
     ed). IRL Press, Washington, D.C.

108

15. Graham, F.L. and Van der Ed, A.J. (1973) A new technique for the assay of human adenovirus 5 DNA, Virology, 52:456.

16. Hatzopoulos, A.K., Schlokat, U., and Gruss, P. (1988) Enhancers and other cis-acting regulatory sequences. In Transcription and Splicing. (Hames, B.D. and Glover, D.M., eds.) IRL Press, Washington, D.C., Vol. 1, p. 43.

17. Hayashi, I., Larner, J., and Sato, G. (1978) Hormonal growth control of cells in culture., In Vitro, 14:23.

18. Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L., and Habener, J.F. (1988) Cyclic AMP-responsive DNA-binding protein: Structure based on a cloned placental cDNA, Science, 242:1430.

19. Hoopes, B.C. and McClure, W.R. Strategies in Regulation of Transcription Initiation. In Escherichia coli and Samonella Typhimurium: Cellular and Molecular Biology (F.C. Neidhardt, J.L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter, eds.) Vol. 2, p. 1231.

20. Kaushansky, K., O'Hara, P.J., Berkner, K., Segal, G.M., Hagen, F.S., and Adamson, J.W. (1986) Genomic cloning, characterization, and multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor, Proc. Natl. Acad. Sci. USA, 83:3101.

21. Krainer, A.R. and Maniatis, T. (1988) RNA splicing.

109

In Transcription and Splicing (Hames, B.D. and
Glover, D.M., eds.) IRL Press, Washington, D.C.,
Vol. 1.

5   22.   La Thangue, N.B. and Rigby, P.W.J. (1988)
          Transacting protein factors and the regulation of
          eukaryotic transcription.   In Transcription and
          Splicing (Hames, B.D. and Glover, D.M., eds) IRL
          Press, Washington, D.C., Vol. 1.

10

    23.   Ladner, M.B., Martin, G.A., Noble, J.A., Nikoloff,
          D.N., Tal, R., Kawaski, E.S., and White, T.J.
          (1987) Human CSF-1: gene structure and alternative
          splicing of mRNA precursors, EMBO J., 6:2693.

15

    24.   Landschulz, W.H., Johnson, P.F., and McKnight, S.L.
          (1988).   The leucine zipper: A hypothetical
          structure common to a new class of DNA binding
          proteins, Science, 240:1759.

20

    25.   Lefevre, C., Imagawa, M., Dana, S., Grindlay, J.,
          Bodner, M., and Karin, M. (1987) Tissue-specific
          expression of the human growth hormone gene is
          conferred in part by the binding of a specific
25        trans-acting factor, EMBO J., 6:971.

    26.   Levine, M. and Hoey, T. (1988) Homeobox proteins as
          sequence-specific transcription factors, Cell,
          55:537.

30

    27.   Lin, F.K., Suggs, S., Lin, C.H, Browne, J.K.,
          Smalling, R. Egrie, J.C., Chen, K.K., Fox, G.M.,
          Martin, F., Stabinsky, Z., Badrawi, S.M., Lai,
          P.H., and Goldwasser, E. (1985) Cloning and
35        expression of the human erythropoietin gene, Pro.

110

Natl. Acad. Sci. USA, 82:7580.

28.  Maniatis, T., Goodbourn, S. and Fischer, J.A.
     (1987) Regulation of inducible and tissue-specific
     gene expression, Science, 236:1237.

29.  Matthews, B.W. (1987) Cro repressor structure and
     its interaction with DNA. In DNA: Protein
     Interactions and Gene Regulation (E.B. Thompson and
     J. Papaconstantinou, eds.) University of Texas
     Press, Austin.

30.  McClure (1985) Ann. Rev. Biochem., 54:171.

31.  McKnight, S.L. (1982) Functional relationships
     between transcriptional control signals of the
     thymidine kinase gene of herpes simplex virus,
     Cell, 31:355.

32.  Nagata, S., Tsuchiya, M., Asano, S., Yamamoto, O.,
     Hirata, Y., Kubota, N., Oheda, M., Nomura, H. and
     Yamazaki, T. (1986) The chromosomal gene structure
     and two mRNAs for human granulocyte
     colony-stimulating factor, EMBO J., 5:575.

33.  Ow, D.W., Wood, K.U, Deluca, M, Dewet, J.R.,
     Melinski, D., and Howell, S.H. Science 234:856-859

34.  Pouwels, Ph.H., Enger-Valk, B.E., and Brammar, W.J.
     (1985) Cloning Vectors. Elsevier Science
     Publishers, B.V., Amsterdam.

35.  Proudfoot, N.J. and Whitelaw, E. (1988) Termination
     and 3' end processing of eukaryotic RNA. In
     Transcription and Splicing (Hames, B.D. and Glover,

D.M., eds.) ERL Press, Washington, D.C., Vol. 1, p.
97.

36.    Schlief, R. The L-Arabinose Dperon. In _Escherichia
       coli_ and _Salmonella typhimurium_: Cellular and
       Molecular Biology (F.C. Neidhardt, J.L. Ingraham,
       K. Brooks Low, B. Maga Sanik, M. Schaecter, eds.)
       Vol. 2, p. 1473.

37.    Schlief, R. (1988) DNA binding by proteins,
       Science, 241:1182.

38.    Yamamoto, K.R. (1985) Steroid receptor regulated
       transcription of specific genes and gene networks,
       Ann. Rev., Genet., 19:209.

39.    Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H.,
       and Montminy, M.R. (1988) Phosphorylation-induced
       binding and transcriptional efficacy of nuclear
       factor CREB, Nature, 334:494.

40.    Yang, Y.C., Ciarletta, A.B., Temple, P.A., Chung,
       M.P., Kovacic, S., Witek-Gianotti, J.S., Learyn,
       A.C., Kriz, R., Donahue, R.E., Wong, G.G., and
       Clark, S.C. (1986) Human IL-3 (multi-CSF):
       Identification by expression cloning of a novel
       hematopoietic growth factor related to murine IL-3,
       Cell, 47:3.

41.    Yang, T.C. and Clark, S.C. (1988) Molecular cloning
       of a primate cDNA and the human gene for
       interleukin 3, Lymphokines, 15:375.

42.    Yanofsky, C. and Crawford, I.P. The Tryptophan
       Operon. In _Escherichia coli_ and _Salmonella_

112

typhimurium: Cellular and Molecular Biology (F.C.
Neidhardt, J.L. Ingraham, K. Brooks Low, B.
Magasanik, M. Schaechter, eds.) Vol. 2, p. 1453.

5      43.   Zhan, A., Culpepper, A., Reddy, M., Loveless, J.,
            and Goldfarb, M. (1987) Human oncogenes detected by
            a defined medium culture assay, Oncogene, 1:369.

       44.   Thomas, K.R. and Capecchi, M.R., Cell, 1987,
10           51:503-512

       45.   Mansour, S.L., Thomas, K.R. and Capecchi, M.R.,
            Nature, 1988, 366:348-352

15     46.   M.R. Capecchi, 1989, Science, 244:1288-1292

       47.   J. Thompson and D. Gillespie (1987) Molecular
            hybridization with RNA probes in concentrated
            solutions of guanidine thiocyanate, Anal. Biochem.
20           163:281-291.

       48.   J. Thompson, R. Solomon, M. Pellegrino, K. Sakai,
            M. Lewin, M. Feild, M. Castrovinci, L. Sacramone
            and D. Gillespie (1989) A noise-free molecular
25           hybridization procedure for measuring RNA in cell
            lysates, Anal. Biochem. 181:371-378.

       49.   M.S. Urdea, J.A. Running, T. Horn, J. Clyne, L. Ku
            and B.D. Warner (1987) A novel method for the rapid
30           detection of specific nucleotide sequences in crude
            biological samples without blotting or
            radioactivity; application to the analysis of
            hepatitis B virus in human serum, GENE 61:253-264.

35     50.   B. Chu and L. Orgel (1989) Target nucleic acid

113

amplification/detection systems, WO 90/03445, published April 5, 1990. Applicant: The Salk Institute for Biological Studies.

5    51.  E. Oura (1983) "Biomass from Carbohydrates" In Biotechnology, Vol 3, Chpt. 1a (ed H. Dellweg) VCH Press, Germany.

     52.  A. Einsele (1983) "Biomass from Higher n-Alkanes" 10   In Biotechnology, Vol 3, Chpt. 1b (ed H. Dellweg) VCH Press, Germany.

     53.  U. Faust and P. Prave (1983) "Biomass from Methane and Methanol" In Biotechnology, Vol 3, Chpt. 1c (ed 15   H. Dellweg) VCH Press, Germany.

     54.  R.J. Quinlan and S.G. Lisansky (1983) "Microbial Insectidides" In Biotechnology, Vol 3, Chpt. 2e (ed H. Dellweg) VCH Press, Germany.
20

     55.  N. Kosaric, A. Wieczorek, G.P. Cosentino, R.J. Magee and J.E. Prenosil (1983) "Ethanol Fermentation" In Biotechnology, Vol 3, Chpt. 3a (ed H. Dellweg) VCH Press, Germany.
25

     56.  M. Rohr, C.P. Kubicek and J. Kominek (1983) "Citric Acid" In Biotechnology, Vol 3, Chpt. 3d (ed H. Dellweg) VCH Press, Germany.

30   57.  M. Rohr, C.P. Kubicek and J. Kominek (1983) "Gluconic Acid" In Biotechnology, Vol 3, Chpt. 3e (ed H. Dellweg) VCH Press, Germany.

     58.  K. Soda, H. Tanaka and N. Esaki (1983) "Amino 35   Acids" In Biotechnology, Vol 3, Chpt. 3g (ed H.

Dellweg) VCH Press, Germany.

59.   G.W. Barnard and D.O. Hall (1983) "Energy from
      Renewable Resources" In Biotechnology, Vol 3, Chpt.
      4 (ed H. Dellweg) VCH Press, Germany.

60.   A. Kuninaka (1986) "Nucleic Acids, Nucleotides, and
      Related Compounds" In Biotechnology, Vol 4, Chpt. 4
      (eds H. Pape and H.-J. Rehm) VCH Press, Germany.

61.   H. Kleinkauf and H. van Dohren (1986) "Peptide
      Antibiotics" In Biotechnology, Vol 4, Chpt. 10 (eds
      H. Pape and H.-J. Rehm) VCH Press, Germany.

62.   S. Umezawa, S. Kondo and Y. Ito (1986)
      "Aminoglycoside Antibiotics" In Biotechnology, Vol
      4, Chpt. 11 (eds H. Pape and H.-J. Rehm) VCH Press,
      Germany.

63.   S. Omura and Y. Tanaka (1986) "Macrolide
      Antibiotics" In Biotechnology, Vol 4, Chpt. 12 (eds
      H. Pape and H.-J. Rehm) VCH Press, Germany.

64.   Z. Hostalek and Z. Vanek (1986) "Tetracyclines" In
      Biotechnology, Vol 4, Chpt. 13 (eds H. Pape and H.-
      J. Rehm) VCH Press, Germany.

65.   G. Lancini (1986) "Ansamycins" In Biotechnology,
      Vol 4, Chpt. 14 (eds H. Pape and H.-J. Rehm) VCH
      Press, Germany.

66.   J. Berdy (1986) "Further Antibiotics with Practical
      Application" In Biotechnology, Vol 4, Chpt. 15 (eds
      H. Pape and H.-J. Rehm) VCH Press, Germany.

115

67. H. Kobel and J.-J. Sanglier (1986) "Ergot Alkaloids" In Biotechnology, Vol 4, Chpt. 18 (eds H. Pape and H.-J. Rehm) VCH Press, Germany.

68. T. Anke (1986) "Further Secondary Products of Biotechnological Interest" In Biotechnology, Vol 4, Chpt. 19 (eds H. Pape and H.-J. Rehm) VCH Press, Germany.

69. J. Berlin (1986) "Secondary Products from Plant Cell Cultures" In Biotechnology, Vol 4, Chpt. 20 (eds H. Pape and H.-J. Rehm) VCH Press, Germany.

70. L.L. Smith (1984) "Steroids" In Biotechnology, Vol 6a, Chpt. 2 (ed K. Kieslich) VCH Press, Germany.

71. C.K.A. Martin (1984) "Sterols" In Biotechnology, Vol 6a, Chpt. 3 (ed K. Kieslich) VCH Press, Germany.

72. V.A. Krasnobajew (1984) "Terpenoids" In Biotechnology, Vol 6a, Chpt. 4 (ed K. Kieslich) VCH Press, Germany.

73. A. Kergomard (1984) "Alicyclic and Heteroalicyclic Compounds" In Biotechnology, Vol 6a, Chpt. 5 (ed K. Kieslich) VCH Press, Germany.

74. O.K. Sebek (1984) "Antibiotics" In Biotechnology, Vol 6a, Chpt. 7 (ed K. Kieslich) VCH Press, Germany.

75. P.R. Wallnofer and G. Engelhardt (1984) "Aromatic and Heterocyclic Structures" In Biotechnology, Vol 6a, Chpt. 8 (ed K. Kieslich) VCH Press, Germany.

76. M. Buhler and J. Schindler (1984) "Aliphatic Hydrocarbons" In Biotechnology, Vol 6a, Chpt. 9 (ed K. Kieslich) VCH Press, Germany.

77. G. Schmidt-Kastner and P. Egerer (1984) "Amino Acids and Peptides" In Biotechnology, Vol 6a, Chpt. 10 (ed K. Kieslich) VCH Press, Germany.

78. A. Crueger and W. Crueger (1984) "Carbohydrates" In Biotechnology, Vol 6a, Chpt. 11 (ed K. Kieslich) VCH Press, Germany.

79. P.F. Heinstein and A. Emery (1988) "Processes with Plant Cell Cultures" In Biotechnology, Vol 6b, Chpt. 9 (ed H.-J. Rehm) VCH Press, Germany.

80. M. Butler (1988) "Processes with Animal Cell and Tissue Cultures" In Biotechnology, Vol 6b, Chpt 10 (ed H.-J. Rehm) VCH press, Germany.

81. A.E. Torma (1988) "Biosensors and 'Bioelectronics'" In Biotechnology, Vol 6b, Chpt 12 (ed H.-J. Rehm) VCH press, Germany.

82. S. Kunst and K. Mudrack (1988) "Microbial Elimination of Nitrogen and Phosphorus" In Biotechnology, Vol 6b, Chpt 14 (ed H.-J. Rehm) VCH press, Germany.

83. E.-J. Nyns (1986) "Biomethanation Processes" In Biotechnology, Vol 8, Chpt 5 (ed W. Schonborn) VCH press, Germany.

84. T. Leisinger and W. Brunner (1986) "Poorly Degradable Substances" In Biotechnology, Vol 8,

117

Chpt 14 (ed W. Schonborn) VCH press, Germany.

85. S. Lafon-Lafourcade (1983) "Wine and Brandy" In
    Biotechnology, Vol 5, Chpt 2 (ed G. Reed) VCH
    press, Germany.

86. W.A. Hardwick (1983) "Beer" In Biotechnology, Vol
    5, Chpt 3 (ed G. Reed) VCH press, Germany.

87. E.R. Vedamuthu and C. Washam (1983) "Cheese" In
    Biotechnology, Vol 5, Chpt 4 (ed G. Reed) VCH
    press, Germany.

88. V. Bottazzi (1983) "Other Fermented Dairy Products"
    In Biotechnology, Vol 5, Chpt 5 (ed G. Reed) VCH
    press, Germany.

89. D.W. Ow, K.V. Wood, M. DeLuca, J.R. DeWet, D.R.
    Helinski, S.H. Howell (1986) Transient and stable
    expression of the firefly luciferase gene in plant
    cells and transgenic plants.  Science 234:836-839.

90. D.R. Edwards and J.K. Heath (1990) "Regulation of
    transcription by transforming growth factor-$\beta$" In
    Molecular Aspects of Cellular Regulation, Vol 6,
    Elsevier Biomedical Press, in press.

91. Transcription and Splicing (1988) In the series
    Frontiers in Molecular Biology, ed. B.D. Hames and
    D.M. Glover, IRL Press, UK.

92. Southern, E. (1980) Methods in Enzymology,
    69:152.

93. Feinberg, A. and Vogelstein (1984) Anal. Biochem.

118

__137__:266.

94. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning. Cold Spring Harbor Laboratory Press, __1__:7.58.

95. Cole, S. P. C. (1986) Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemother. Pharmacol., __17__:259.

96. Harlow, E. and Lane, D. (1988). Antibodies - A Laboratory Manual. Cold Spring Harbor Laboratory Press, page 578.

97. Teem, J.L. and Rosbash, M. (1983) Proc. Natl. Acad. Sci. USA 80:4403-4407.

98. Pikielny, C.W., Teem, J.L., and Rosbash, M. (1983) Cell 34:395-403.

99. Sherman, F., Fink, G.R., Hicks, J.B. (1986) Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

100. DeWet, et al., (1987) Mol. Cell. Biol. 7:725-737.

What is claimed is:

1.  A method of transcriptionally modulating the
    expression of a homologous gene-of-interest, the
    expression of which is associated with a defined
    physiological or pathological effect within a
    multicellular organism, which comprises contacting
    a cell, which is capable of expressing the gene,
    with an amount of a molecule effective to
    transcriptionally modulate expression of the gene
    and thereby affect the level of the protein encoded
    by the gene which is expressed by the cell, which
    molecule (a) does not naturally occur in the cell,
    (b) specifically transcriptionally modulates
    expression of the gene-of-interest, and (c) binds
    to DNA or RNA, or binds to a protein through a
    domain of such protein which is not a
    ligand-binding domain of a receptor which naturally
    occurs in the cell, the binding of a ligand to
    which ligand-binding domain is normally associated
    with the defined physiological or pathological
    effect.

2.  A method of claim 1, wherein the molecule does not
    naturally occur in any cell of a higher eucaryotic
    organism.

3.  A method of claim 1, wherein the molecule does not
    naturally occur in any cell.

4.  A method of claim 1, wherein the molecule is not a
    naturally occurring molecule.

5.  A method of claim 1, wherein the cell is a cell of

the multicellular organism.

6.   A method of claim 1, wherein the cell is an animal
     cell.

7.   A method of claim 6, wherein the animal cell is a
     human cell.

8.   A method of claim 1, wherein the cell is a plant
     cell.

9.   A method of claim 1, wherein the cell is a fungal
     cell.

10.  A method of claim 1, wherein the cell is a
     protozoan cell.

11.  A method of claim 1, wherein the cell is a
     bacterial cell.

12.  A method of claim 1, wherein the gene-of-interest
     is a human gene.

13.  A method of claim 1, wherein the gene-of-interest
     encodes a hematopoietic protein.

14.  A method of claim 13, wherein the hematopoietic
     protein is a colony stimulating factor.

15.  A method of claim 14, wherein the colony
     stimulating factor is granulocyte-macrophage colony
     stimulating factor (GM-CSF).

16.  A method of claim 14, wherein the colony

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stimulating    factor    is    granulocyte    colony
stimulating factor (G-CSF).

17.    A    method    of    claim    14,    wherein    the    colony
      stimulating factor is macrophage colony stimulating
      factor (M-CSF).

18.    A method of claim 13, wherein the hematopoietic
      protein is erythropoietin (EPO).

19.    A method of claim 1, wherein the gene-of-interest
      encodes an interleukin (IL).

20.    A method of claim 1, wherein the gene-of-interest
      encodes a growth hormone selected from the group
      consisting of human, bovine, porcine, avian, ovine,
      piscine, and equine growth hormone, and polypeptide
      analogs thereof having the biological activity of
      the    corresponding    naturally    occurring    growth
      hormone.

21.    A method of claim 1, wherein the gene of interest
      is a growth hormone releasing factor.

22.    A method of claim 1, wherein the gene-of-interest
      is a viral gene.

23.    A method of claim 22, wherein the viral gene is a
      retroviral gene.

24.    A method of claim 23, wherein the retroviral gene
      is a gene from the HIV, HTLV-1, or HTLV-2 virus.

25.    A method of claim 22, wherein the viral gene is a

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gene from a hepatitis virus.

26.   A method of claim 22, wherein the viral gene is a
      gene from a herpes virus.

27.   A method of claim 22, wherein the viral gene is a
      gene from an animal virus.

28.   A method of claim 22, wherein the viral gene is a
      gene from a papilloma virus.

29.   A method of claim 22, wherein the viral gene is a
      gene from a cytomegalovirus.

30.   A method of claim 27, wherein the animal virus is a
      pseudorabies, Marek's, Newcastle's Disease, or IBR
      virus.

31.   A method of claim 1, wherein the gene-of-interest
      is a plant gene.

32.   A method of claim 31, wherein the plant gene
      encodes an agronomically important trait.

33.   A method of claim 32, wherein the agronomically
      important trait is selected from the group
      consisting of germination, sprouting, flowering,
      fruit ripening, salt tolerance, herbicide
      resistance, pesticide resistance, fungicide
      resistance, temperature resistance, and growth.

34.   A method of claim 1, wherein the gene-of-interest
      is a protozoan gene.

35.   A method of claim 34, wherein the protozoan is

selected from the group consisting of _Trypanosoma_,
_Plasmodium_, _Leishmania_, _Giardia_, _Entamoeba_,
_Toxoplasma_, _Babesia_, and _Cryptosporidiosis_.

36. A method of claim 1, wherein the gene-of-interest
    is a helminth gene.

37. A method of claim 1, wherein the gene-of-interest
    is an oncogene.

38. A method of claim 37, wherein the oncogene is the
    phl-_abl_ oncogene.

39. A method of claim 37, wherein the oncogene is
    selected from the group consisting of H-, N-, and
    K-_ras_ oncogenes.

40. A method of claim 37, wherein the oncogene is the
    _neu_ oncogene.

41. A method of claim 37, wherein the oncogene is the
    _src_ oncogene.

42. A method of claim 1, wherein the gene-of-interest
    encodes TGF-ß1.

43. A method of claim 1, wherein the gene-of-interest
    encodes TGF-ß2.

44. A method of claim 1, wherein the gene-of-interest
    encodes TGF-ß3.

45. A method of claim 1, wherein the gene-of-interest
    encodes a naturally occurring receptor.

46.    A method of claim 45 wherein the receptor is a
       receptor for a TGF-ß.

47.    A method of claim 45, wherein the receptor is a
       testosterone receptor.

48.    A method of claim 45, wherein the receptor is an
       estrogen receptor.

49.    A method of claim 45, wherein the receptor is the
       human low density lipoprotein (LDL) receptor.

50.    A method of claim 45, wherein the receptor is the
       receptor for a hematopoietic protein selected from
       the group consisting of M-CSF, G-CSF, GM-CSF, and
       EPO.

51.    A method of claim 45, wherein the receptor is the
       receptor for an interleukin (IL) selected from the
       group consisting of IL-1, IL-2, IL-3, IL-4, IL-5,
       IL-6, IL-7 and IL-8.

52.    A method of claim 45, wherein the receptor is a
       cell surface protein which mediates infection of
       the cell by a virus.

53.    A method of claim 52, wherein the virus is selected
       from the group consisting of HIV, HTLV-1, HTLV-2, a
       hepatitis virus, a herpes virus, an animal virus, a
       papilloma    virus,    a    cytomegalovirus,    and    a
       rhinovirus.

54.    A method of claim 1, wherein the receptor which
       naturally occurs in the cell is a testosterone
       receptor.

55. A method of claim 1, wherein the receptor which naturally occurs in the cell is an estrogen receptor.

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56. A method of claim 1, wherein in (c) the protein is not the protein encoded by the gene-of-interest.

57. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene, which expresses a polypeptide capable of producing a detectable signal, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measureable detectable signal to be produced by the polypeptide expressed by the reporter gene, quantitatively determining the amount of the signal produced, comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene, and thus identifying the molecule as a molecule capable of transcriptionally modulating

the expression of the gene-of-interest.

58.   A method of determining whether a molecule not
      previously known to be a modulator of protein
5     biosynthesis is capable of transcriptionally
      modulating the expression of a gene-of-interest
      which comprises contacting a sample which contains
      a predefined number of cells with a predetermined
      amount of a molecule to be tested, each such cell
10    comprising DNA consisting essentially of (i) a
      modulatable transcriptional regulatory sequence of
      the gene-of-interest, (ii) a promoter of the gene-
      of-interest, and (iii) a DNA sequence transcribable
      into mRNA coupled to and under the control of, the
15    promoter, under conditions such that the molecule,
      if capable of acting as a transcriptional modulator
      of the gene-of-interest, causes a measureable
      difference in the amount of mRNA transcribed from
      the DNA sequence, quantitatively determining the
20    amount of the mRNA produced, comparing the amount
      so determined with the amount of mRNA detected in
      the absence of any molecule being tested or upon
      contacting the sample with any other molecule, and
      thereby identifying the molecule as one which
25    causes a change in the amount of detectable mRNA
      amount of, and thus identifying the molecule as a
      molecule capable of transcriptionally modulating
      the expression of the gene-of-interest.

30    59.   A method of any of claims 57 or 58, wherein the
      molecule (a) does not naturally occur in the cell,
      (b)   specifically   transcriptionally   modulates
      expression of the gene-of-interest, and (c) binds
      to DNA or RNA or binds to a protein through a
35    domain of such protein which is not a ligand

binding domain of a receptor which naturally occurs
in the cell, the binding of a  ligand to which
ligand binding domain is normally associated with a
defined physiological or pathological effect.

60.  A method of any of claims 57 or 58, wherein the
     sample comprises cells in monolayers.

61.  A method of any of claims 57 or 58, wherein the
     sample comprises cells in suspension.

62.  A method of any of claims 57 or 58, wherein the
     cells comprise human, animal, or plant cells.

63.  A method of any of claims 57 or 58, wherein the
     cells are bacterial cells.

64.  A method of any of claims 57 or 58, wherein the
     cells are fungal cells.

65.  A method of any of claims 57 or 58, wherein the
     predefined number of cells is from about 1 to about
     $5 \times 10^5$ cells.

66.  A method of claim 65, wherein the predefined number
     of cells is from about $2 \times 10^2$ to about $5 \times 10^4$
     cells.

67.  A method of any of claims 57 or 58, wherein the
     predetermined amount of the molecule to be tested
     is based upon the volume of the sample.

68.  A method of any of claims 57 or 58, wherein the
     predetermined amount is from about 1.0pM to about
     20 $\mu$M.

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69. A method of any of claims 57 or 58, wherein the predetermined amount is from about 10nM to about 500μM.

70. A method of any of claims 57 or 58, wherein the contacting is effected from about 1 hour to about 24 hours.

71. A method of claim 70, wherein the contacting is effected from about 2 to about 12 hours.

72. A method of any of claims 57 or 58, wherein the contacting is effected with more than one predetermined amount of the molecule to be tested.

73. A method of any of claims 57 or 58, wherein the molecule to be tested is a purified molecule.

74. A method of any of claims 57 or 58, wherein the modulatable transcriptional regulatory sequence comprises a cloned genomic regulatory sequence.

75. A method of any of claims 57 or 58, wherein the DNA consists essentially of more than one modulatable transcriptional regulatory sequence.

76. A method of claim 57, wherein the reporter gene is inserted downstream of the promoter of the gene-of-interest by homologous recombination.

77. A method of claim 57, wherein the reporter gene encodes a luciferase.

78. A method of claim 57, wherein the reporter gene encodes chloramphenicol acetyltransferase.

79.   A method of claim 57, wherein the reporter gene encodes β glucuronidase.

80.   A method of claim 57, wherein the reporter gene encodes β galactosidase.

81.   A method of claim 57, wherein the reporter gene encodes neomycin phosphotransferase.

82.   A method of claim 57, wherein the reporter gene encodes guanine xanthine phosphoribosyltransferase.

83.   A method of claim 58, wherein mRNA is detected by quantitative polymerase chain reaction.

84.   A screening method according to any of claims 57 or 58 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells, under conditions such that contacting is effected with a predetermined amount of each different molecule to be tested.

85.   A screening method of claim 84, wherein the plurality of samples comprises more that about $10^6$ samples.

86.   A screening method of claim 84, wherein the plurality of samples comprises more than about 6 X $10^6$ samples.

87.   A method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes of interest in a panel of such genes which

130

comprises essentially simultaneously screening the
molecules against each of the genes of interest
according to the method of claim 84.

88.  A screening method of any of claims 86 or 87,
     wherein more that about $10^3$ samples per week are
     contacted with different molecules.

89.  A method for transcriptionally modulating in a
     multicellular organism the expression of a gene-of-
     interest, the expression of which is associated
     with a defined physiological or pathological effect
     in the organism, which comprises administering to
     the organism an amount of a molecule effective to
     transcriptionally modulate expression of the gene
     and thus affect the defined physiological or
     pathological effect, which molecule (a) does not
     naturally occur in the organism, (b) specifically
     transcriptionally modulates expression of the gene-
     of-interest, and (c) binds to DNA or RNA or binds
     to a protein through a domain of such protein which
     is not a ligand binding domain of a receptor which
     naturally occurs in the organism, the binding of a
     ligand to which ligand binding domain is normally
     associated with the defined physiological or
     pathological effect.

90.  A method of claim 89, wherein the multicellular
     organism is a human being.

91.  A method of claim 89, wherein the multicellular
     organism is an animal.

92.  A method of claim 89, wherein the multicellular organism is a plant.

93.  A method of claim 89, wherein the defined pathological effect is a disorder and modulated expression of the gene-of-interest is associated with amelioration of the disorder.

94.  A method of claim 90, wherein the defined pathological effect is a disorder selected from the group consisting of cancer, a hematopoietic dysfunction, diabetes, tissue inflammation, atherosclerosis, viral infections, dysfunctions of memory or learning, and dysfunctions in a cholesterol or other metabolic pathway.

95.  A method of claim 91, wherein the defined physiological effect is growth and the organism is an animal such as a man, a cow, a pig, a bird, a fish, a sheep or a horse.

96.  A method of claim 92, wherein the defined physiological or pathological effect is an agronomically important trait.

97.  A method of claim 90 or 91, wherein the administering comprises topical contact.

98.  A method of claim 90 or 91, wherein the administering comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration.

99.  A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes a naturally occurring

receptor.

100. A method of claim 99, wherein the receptor is a
     testosterone receptor.

101. A method of claim 99, wherein the receptor is an
     estrogen receptor.

102. A method of any of claims 57, 58, or 89, wherein
     the receptor which naturally occurs in the cell is
     a testosterone receptor.

103. A method of any of claims 57, 58, or 89, wherein
     the receptor which naturally occurs in the cell is
     an estrogen receptor.

104. A method of any of claims 57, 58, or 89, wherein
     the gene-of-interest encodes a TGF-ß receptor.

105. A method of any of claims 57, 58, or 89, wherein
     the gene-of-interest encodes TGF-ß1.

106. A method of any of claims 57, 58, or 89, wherein
     the gene-of-interest encodes TGF-ß2.

107. A method of any of claims 57, 58, or 89, wherein
     the gene-of-interest encodes TGF-ß-3.

108. A method of any of claims 57, 58, or 89, wherein
     the gene-of-interest encodes an oncogene.

109. A method of claim 108, wherein the oncogene is the
     neu oncogene.

110. A method of claim 108, wherein the oncogene is

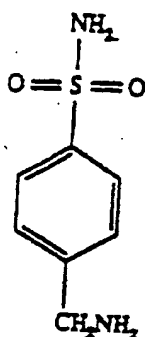selected from the group consisting of H-, N-, and
K-ras oncogenes.

111. A method for enhancing the expression of human
growth hormone by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing, human
growth hormone, comprising contacting the cell with
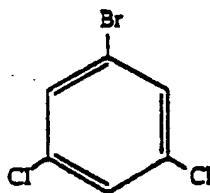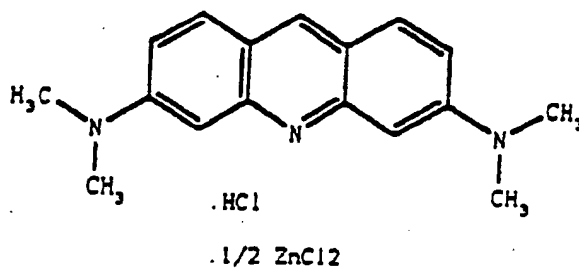an amount of a molecule having the structure:

effective to enhance the expression of human
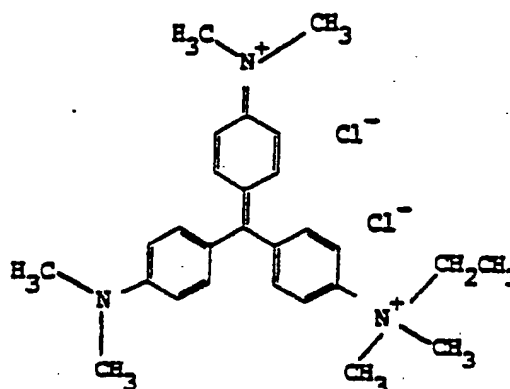growth hormone by the cell.

112. A method for enhancing the expression of human
growth hormone by a cell which (i) comprises DNA
encoding, and (ii) is capable of expressing
contacting the cell with an amount of a molecule
having the structure:

134

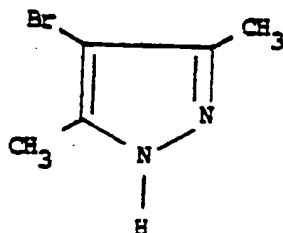effective to enhance the expression of human
growth hormone by the cell.

113. A method for enhancing the expression of human
     growth hormone by a cell which (i) comprises DNA
     encoding, and (ii) is capable of expressing, human
     growth hormone, comprising contacting the cell with
     an amount of a molecule having the structure:



effective to enhance the expression of human
growth hormone by the cell.

114. A method for enhancing the expression of human
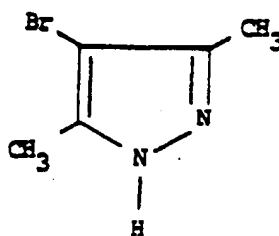     growth hormone by a cell which (i) comprises DNA
     encoding, and (ii) is capable of expressing, human
     growth hormone, comprising contacting the cell with
     an amount of a molecule having the structure:

effective to enhance the expression of human growth
hormone by the cell.

115. A method for enhancing the expression of human
     growth hormone by a cell which (i) comprises DNA
     encoding, and (ii) is capable of expressing, human
     growth hormone, comprising contacting the cell with
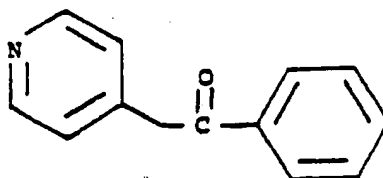     an amount of a molecule having the structure:



     wherein R is
     effective to enhance the expression of human
     growth hormone by the cell.

116. A method for enhancing the expression of G-CSF by a
     cell which (i) is capable of expressing, G-CSF,
     comprising contacting the cell with an amount of a
     molecule having the structure:



     effective to enhance the expression of G-CSF
     by the cell.

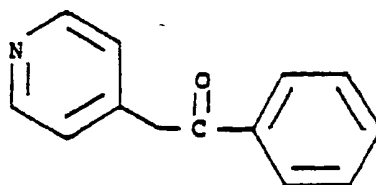117. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of molecule having the
structure:

effective to enhance the expression of G-CSF
by the cell.

118. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

effective to enhance the expression of G-CSF
by the cell.

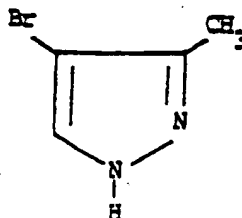119. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

effective to enhance the expression of G-CSF
by the cell.

120. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

wherein R1 is hydrogen or a lower alkyl group,
wherein R2 is hydrogen or a lower alkyl group,
wherein X is oxygen or -NH₂,
wherein Y is bromine, fluorine, chlorine, or
iodine, and
which molecule is effective to enhance the
expression of G-CSF by the cell.

121. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
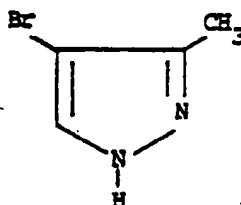the cell with an amount of a molecule having the
structure:

$$\underset{S}{\overset{\displaystyle H_3C\diagdown \underset{\displaystyle \overset{CH_3}{|}}{N}\diagup \underset{\displaystyle \underset{S}{\|}}{C}\diagup \underset{\displaystyle \overset{CH_2CN}{|}}{S}}{}}$$

effective to enhance the expression of G-CSF
by the cell.

122. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
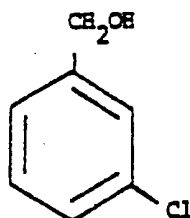the cell with an amount of a molecule having the
structure:

140



effective to enhance the expression of G-CSF
by the cell.

123. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
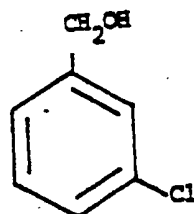capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:



effective to enhance the expression of G-CSF
by the cell.

124. A method for enhancing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:

141

$H_3C$—N—CH$_3$ (acridine orange type structure with dimethylamino groups)

. HCl

. 1/2 ZnCl2

effective to enhance the expression of G-CSF
by the cell.

125. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
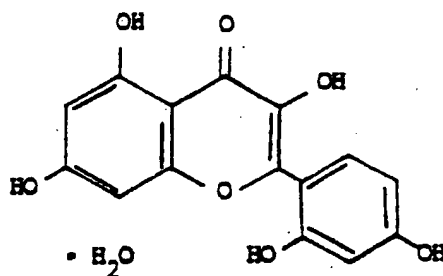the cell with an amount of a  molecule having the
structure:

effective to decrease the expression of G-CSF
by the human being.

126. A method for decreasing the expression of G-CSF by
a cell which (i) comprises DNA encoding, and (ii)

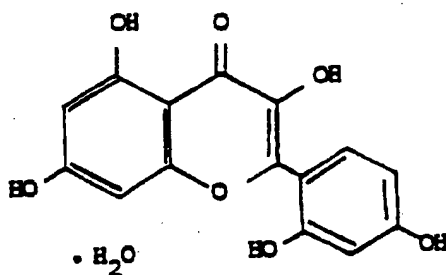142

is capable of expressing, G-CSF, comprising
contacting the cell with an amount of a molecule
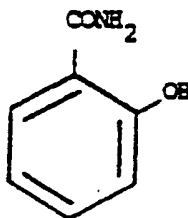having the structure:

5



10

effective to decrease the expression of G-CSF
by the human being.

127. A method of decreasing the formation of neutrophils
     and affecting the metabolic functions of
     neutrophils in a human being who (i) comprises DNA
     encoding, and (ii) is capable of expressing, G-CSF,
     comprising administering to the human being an
     amount of a molecule having the structure:



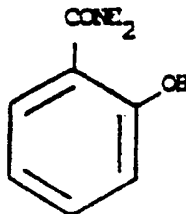     effective to decrease expression of G-CSF by,
     and thus decrease the formation and effect the
     metabolic functions of neutrophils in, the
     human being.

128. A method of decreasing the expression of G-CSF by a
     cell which (i) comprises DNA encoding, and (ii) is
     capable of expressing, G-CSF, comprising contacting
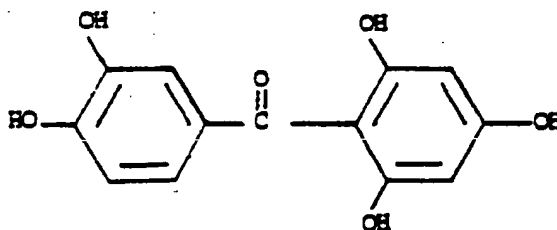     the cell with an amount of a molecule having the
     structure:



     effective to decrease the expression of G-CSF

144

by the human being.

129. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:
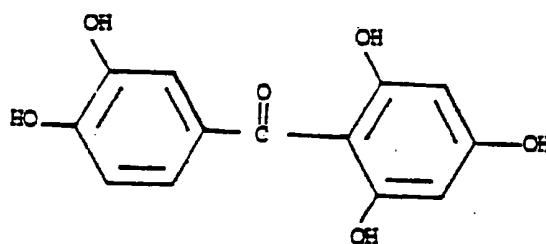


effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

130. A method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:

145

$$\begin{array}{c} Br \qquad\qquad CH_3 \\ \\ N \\ \\ N \\ H \end{array}$$
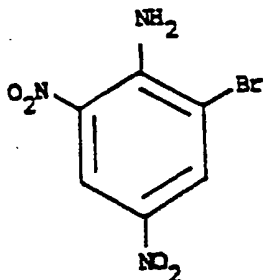
effective to decrease the expression of G-CSF
by the human being.

131. A method of decreasing the formation of neutrophils
and affecting the metabolic functions of
neutrophils in a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an
amount of a molecule having the structure:

$$\begin{array}{c} Br \qquad\qquad CH_3 \\ \\ N \\ \\ N \\ H \end{array}$$

effective to decrease expression of G-CSF by,
and thus decrease the formation and effect the
metabolic functions of neutrophils in, the
human being.

132. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting

146

the cell with an amount of a molecule having the
structure:

$$CH_2OH$$



effective to decrease the expression of G-CSF by
the human being.

133. A method of decreasing the formation of neutrophils
and affecting the metabolic functions of
neutrophils in a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an
amount of a molecule having the structure:

$$CH_2OH$$



effective to decrease expression of G-CSF by, and
thus decrease the formation and effect the
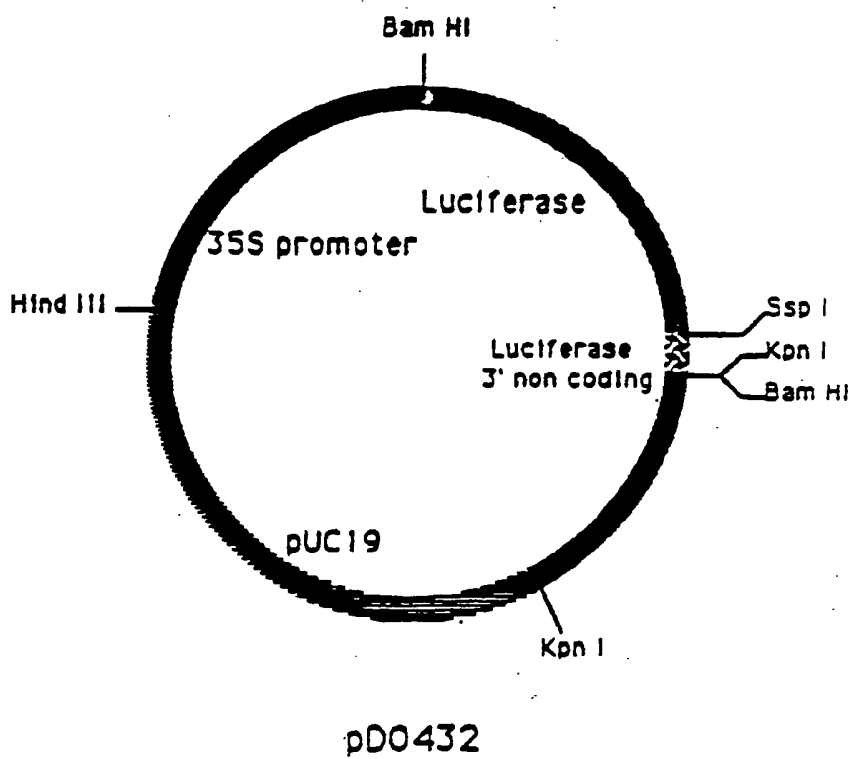metabolic functions of neutrophils in, the human
being.

134. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting

147

the cell with an amount of a molecule having the structure:



· H₂O

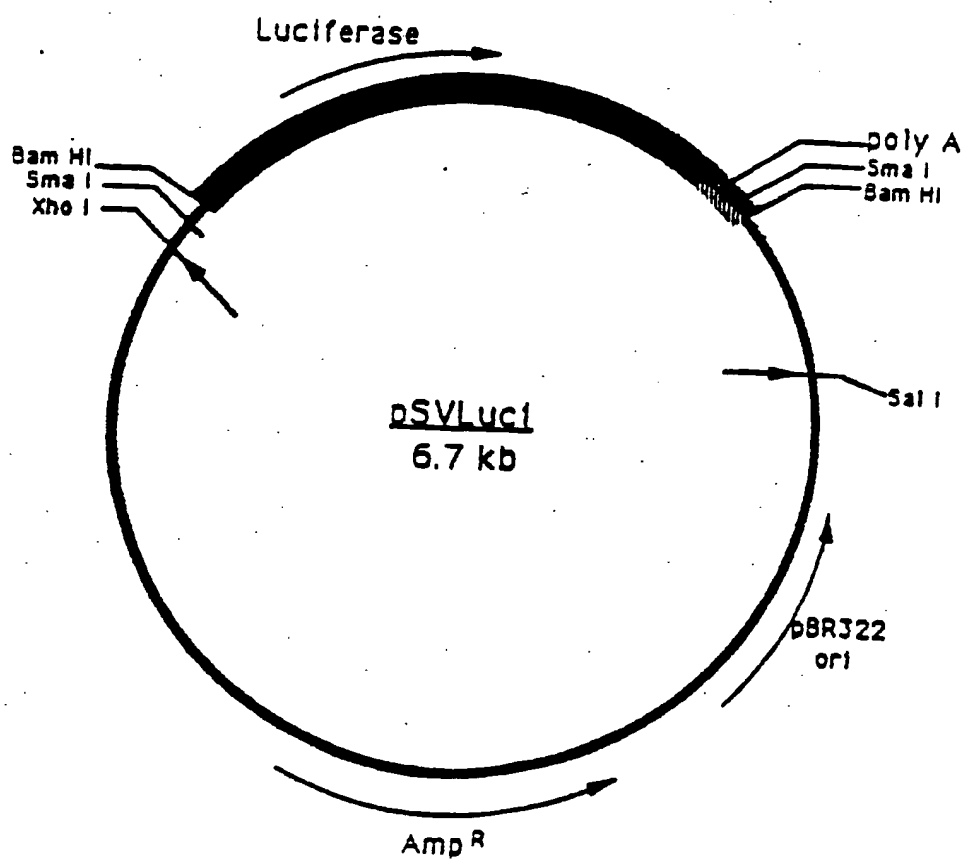effective to decrease the expression of G-CSF by the human being.

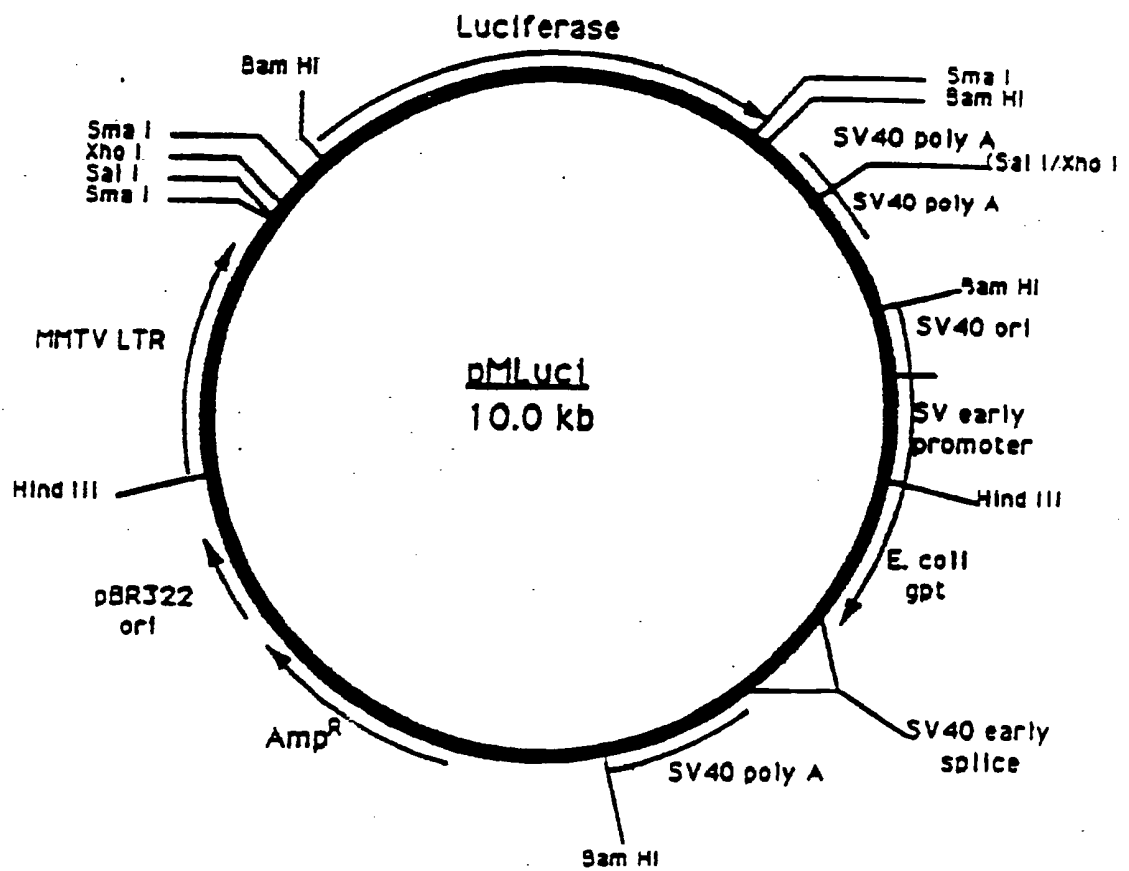135. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



· H₂O

effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

136. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
5       structure:

$$CONH_2$$

OH

10

15

effective to decrease the expression of G-CSF
by the human being.

20   137. A method of decreasing the formation of neutrophils
and    affecting    the    metabolic    functions    of
neutrophils in a human being who (i) comprises DNA
encoding, and (ii) is capable of expressing, G-CSF,
comprising administering to the human being an
25       amount of a molecule having the structure:

$$CONH_2$$

OH

30

35

149

effective to decrease expression of G-CSF by,
and thus decrease the formation and effect the
metabolic functions of neutrophils in, the
human being.

138. A method of decreasing the expression of G-CSF by a
cell which (i) comprises DNA encoding, and (ii) is
capable of expressing, G-CSF, comprising contacting
the cell with an amount of a molecule having the
structure:



effective to decrease the expression of G-CSF
by the human being.

139. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

140. A method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:

effective to decrease the expression of G-CSF
by the human being.

5

Figure 1. pDO432



pDO432

Figure 2. pSVLuc1

Figure 3. pMLuci

Figure 4. pUXLuci

Figure 5. phGH-CAT

Figure 6. phGH-Luc1



pUXLuc1

hGH promoter

Xba I
Sal I
Bam HI

Luciferase

phGH:luc1
5650 bp

ori

SV40 late poly A

Amp^R

## Figure 7. pJM710

Figure 8. pGEM5-Luct

Figure 9. pG-Luc 1

Figure 10. Response of Reporter Cell Clones To Known Inducers
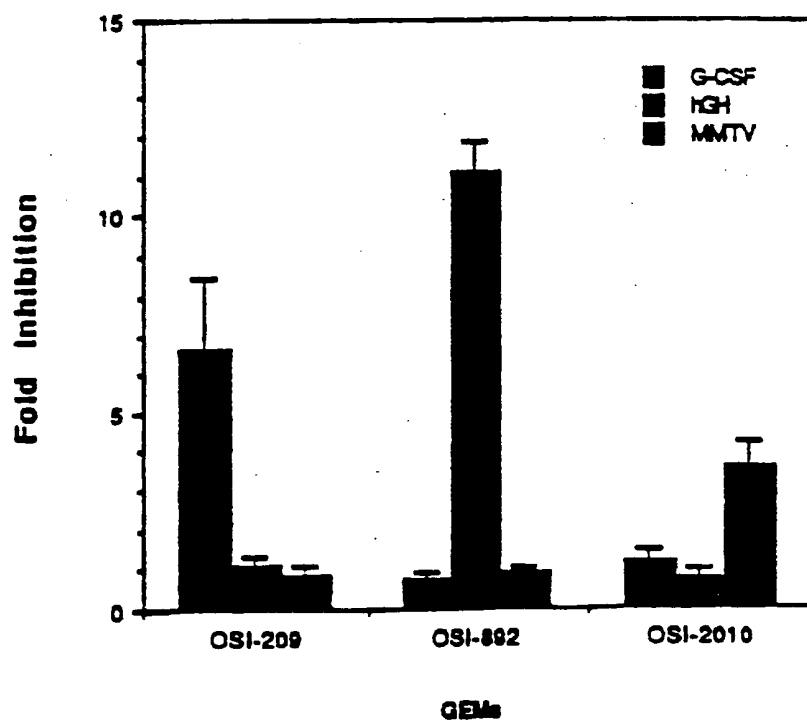
Figure 11 . Effects of Steroids on Clone M10



Light Units/mg prot

Figure 12

EXAMPLES OF PRIMARY SCREEN LEAD CHEMICALS—
SPECIFIC TRANSCRIPTIONAL INDUCERS

Figure 13

EXAMPLES OF PRIMARY SCREEN LEAD CHEMICALS
SPECIFIC TRANSCRIPTIONAL INHIBITORS

Figure 14

Figure 15
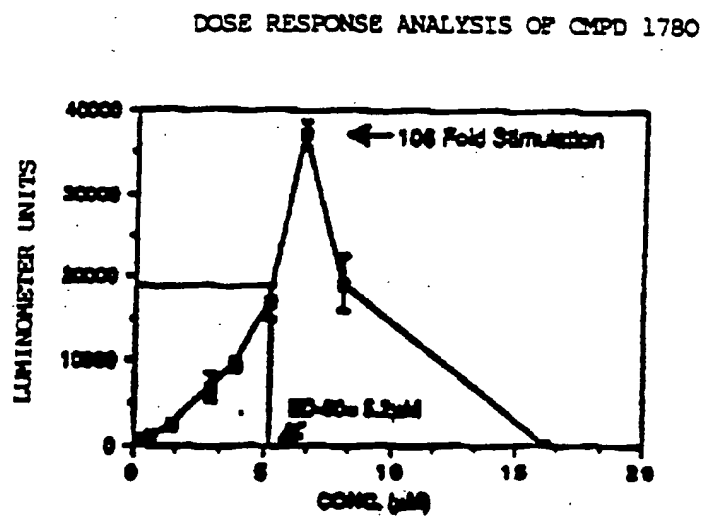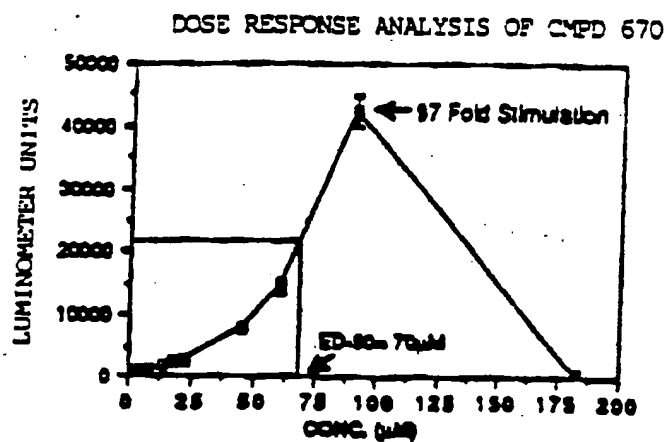
Figure 16

DOSE RESPONSE ANALYSIS OF CMPD 670



DOSE RESPONSE ANALYSIS OF CMPD 1780



DOSE RESPONSE ANALYSIS OF CMPD 80
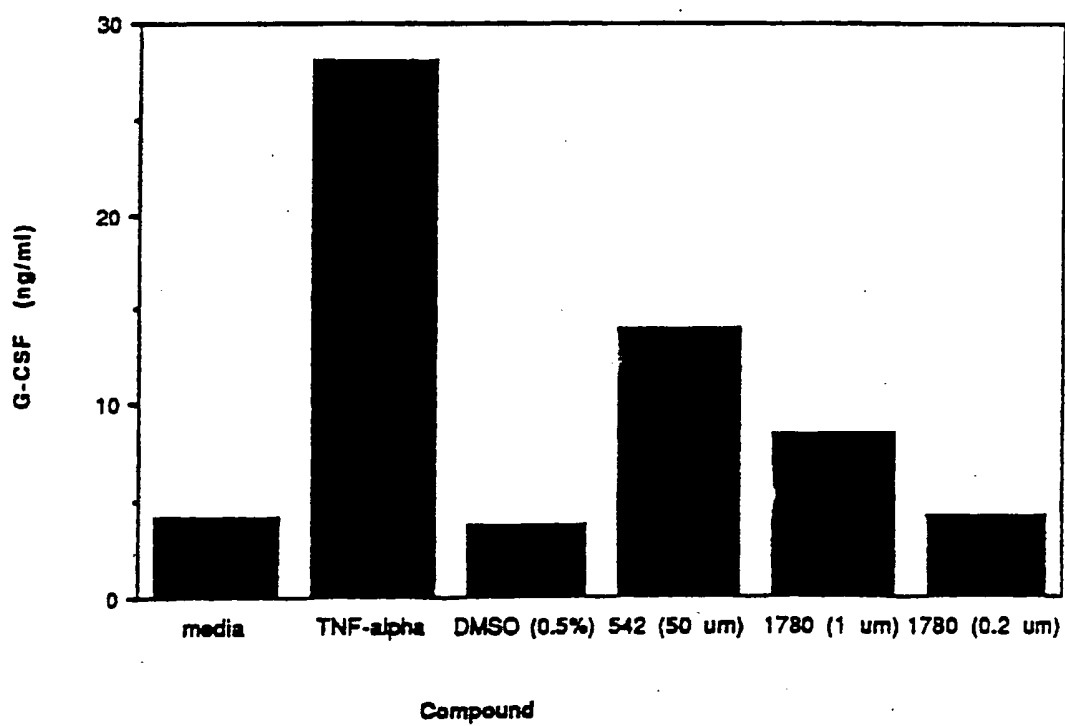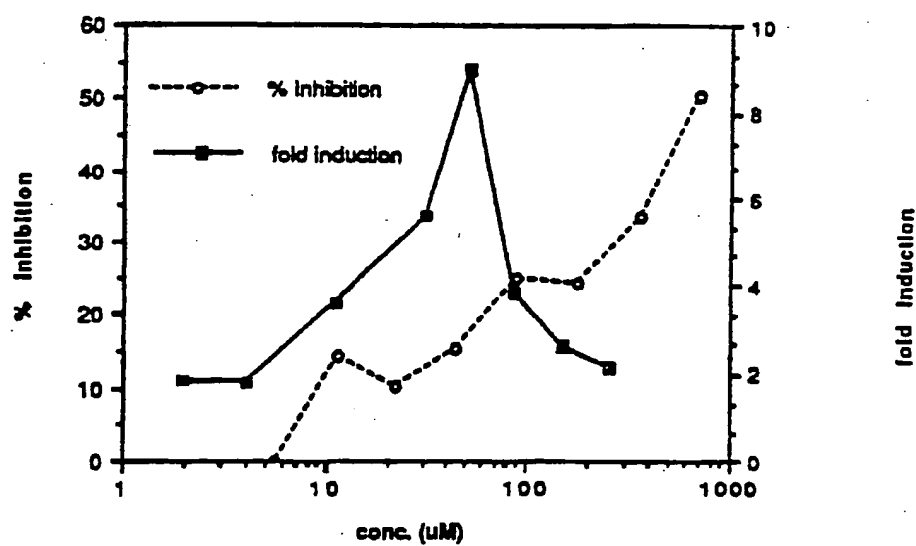
Figure 17

G-CSF concentration in cell supernatant after 48 hours incubation

Figure 18

542 - promoter stimulation versus respiratory inhibition

# INTERNATIONAL SEARCH REPORT

International Application No **PCT/US90/04021**

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 21/00,21/02; C12N 7/04,5/00,15/00,1/38; C12Q 1/66
USA: 435/69.5,69.62,69.4,70.1,236,240.2,172.1,172.2,172.3,244

## II. FIELDS SEARCHED

### Minimum Documentation Searched ⁴

| Classification System | Classification Symbols |
|---|---|
| US | 435/8,69.4,69.5,69.62,70.1,172.1,172.2,172.3,236,240.2,244 |

### Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

U.S. Patent and Trademark Office Automated patent System
DIALOG- BIOSIS PREVIEWS, CHINESE PATENT ABSTRACTS, CLAIMS™/U.S. PATENTS,
INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENTS INDEX (SEE ATTACHMENT)

## III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

| Category * | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ |
|---|---|---|
| X / Y | US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col. 1-10 and the examples. | 1-6 / 1-7,12 |
| X / Y | US, A, 4,740,461 (Kaufman) 26 April 1988. See col. 1-16 and the examples. | 1-6 / 1-7,12 |
| X / Y | EP, A, 0,117,058 (Levinson et al.) 29 August 1984. See at least the abstract and pages 5-18. | 1-6 / 1-7,12 |
| X / Y | Mol. Cell. Biol. Volume 7, No.6, issued June 1987 (Angel et al.) "12-0-tetradecanoyl phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region pages 2256-2266. See the abstract and figures. | 1-7 / 1-7,12 |

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search ² | Date of Mailing of this International Search Report ² |
|---|---|
| 17 October 1990 | 02 JAN 1991 |

| International Searching Authority ¹ | Signature of Authorized Officer ¹⁰ |
|---|---|
| ISA/US | *Christopher Low* Christopher Low |

Form PCT/ISA/210 (second sheet) (May 1986)

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

| | | |
|---|---|---|
| Y | Proc. Natl. Acad. Sci. USA, Volume 83, issued May 1986 (Kaushansky et al.) "Genomic cloning, characterization, multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor". Pages 3101-3105. See Figure 2, and page 3105.<br><br>(See Attachment) | 1-7,12-15 |

**V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]**

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ..... ... because they relate to subject matter[1] not required to be searched by this Authority, namely:

2.☐ Claim numbers............, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out[1], specifically:

3.☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]**

This International Searching Authority found multiple inventions in this International application as follows:

### (See Attachment)

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

### 1-7 and 12-19

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Y           EMBO J. Volume 6, No. 9, issued September 1987   1-7,12-14,
(Ladner et al.) "Human CSF-1: gene structure and    17
alternative splicing of mRNA". pages 2693-2698
See at least page 2693, 2696-2997.

Y           EMBO J. Volume 6, No. 4, issued April 1987     1-7, 12
(Lefevre et al.) "Tissue-specific expression of
the human growth hormone gene is conferred in
part by the binding of a specific trans-acting
factor". pages 971-981. See at least page 971.

Y           Proc. Natl. Acad. Sci. USA. Volume 82, issued   1-7,12,13
November 1985 (Lin et al.) "Cloning and         18
expression of the human erythropoietin gene".
pages 7580-7584. See entire document.

Y           EMBO J. Volume 5, No. 3, issued March 1986,    1-7,12-14
(Nagata et al.) "The chromosomal gene structure   16
and two mRNAs for human granulocyte colony-
stimulating factor. pages 575-581. See at least
the abstract.

Y           Cell Volume 47, issued 10 October 1986, (Yang   1-7,12-14,
et al.) "Human IL-3 (multi-CSF): Identification 17,19
by expression cloning of a novel hematopoietic
growth factor related to murine IL-3. pages
3-10. See Figure 4, and page 6.

Y           de Serres et al., "Chemial Mutagens. Principles 1-7,12-19
and methods for their detection", published
1980, Plenum Press, New York. pages 331, and
365-473.  See at least pages 331, 367-369, 377.

Y           Science Volume 227, issued 15 March 1985,     1-7,12-19
(Engebrecht et al.) "Measuring gene expression
with light". pages 1345-1347. See entire
document.

Y           Science Volume 236, issued 05 June 1987,      1-7,12-19
(Maniatis et al.) "Regulation of inducible
tissue-specific gene expression. pages 1237-1245
See pages 1237, 1239, 1240, 1243.

Y           Molec. Cell. Biol. Volume 7, No. 2, issued    1-7, 12-19
February 1987, (de Wet et al.) "Firefly
luciferase gene: Structure and expression in
mammalian cells". pages 725-737. See the
abstract, Fig. 1 and 3, pages 729-734.

Documentation searched other than minimum documentation ro
the extent that such documents are included in the fields
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Search Terms:

mammal, toxicity, toxic, chemial, chemicals, carcinogen,
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